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Signaling dynamics in rice expressing constitutively active Nicotiana Protein Kinase 1 (NPK1)

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Signaling dynamics in rice expressing constitutively active *Nicotiana* Protein Kinase 1 (NPK1)

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Abstract

Signaling components such as mitogen-activated protein kinases (MAPKs) regulate stress-induced pathways and are potentially a powerful means of genetically engineering plant tolerance to abiotic stress. The kinase domain of tobacco mitogen-activated protein kinase Nicotiana protein kinase 1 (NPK1) into rice cultivar Nipponbare. Four-week old transgenic and wild type plants treated with moderate (150mM) or severe (300mM) salt stress did not differ in visible signs of leaf damage, electrolyte leakage, malondialdehyde content or ion content within leaves. Expression of *caNPK1* alters gene transcript abundance in the absence of stress as assessed by microarray analysis. Genes potentially related to flowering and development are downregulated while biotin biosynthesis and methionine recycling pathway as well as thirteen transcripts with matches to stress-related genes appear upregulated in the transgenic plants. Quantitative real-time PCR analysis indicates no significant difference in expression of several known rice MAPKs between *caNPK1* transgenics and wild type.

Chapter 1: General introduction

Thesis organization

This thesis encompasses work related to rice transformed with a constitutively active form of a tobacco mitogen-activated protein kinase kinase kinase (MAP3K), *Nicotiana* Protein Kinase 1 (NPK1). The aim of my research has been to determine the observable effects upon gene transcription due to introduction of a constitutively active NPK1 (caNPK1) and the possible implications for use of caNPK1 in enhancing abiotic stress tolerance in rice. Chapter 1 presents a general review of genetic engineering strategies being tested to fortify crops against abiotic stress, including published experiments making use of plant MAPKs and *caNPK1* in tobacco and maize (Kovtun *et al.* 1998; 2000; Shou *et al.* 2004a, b). Those data set the stage for the work described in this thesis. Chapter 2 describes the genetic characteristics of the *caNPK1* transgenic rice lines generated by the Plant Transformation Facility. Results obtained from studies of *caNPK1* transgenic and non-transgenic rice subjected to salt stress are presented in Chapter 3. Chapter 4 is formatted as a manuscript in which microarray analysis and qRT-PCR analysis were used to observe the effects of *caNPK1* expression upon gene transcription. Some elements of qRT-PCR analysis remain to be completed at the time of this thesis submission and are thus noted in the Results section of the manuscript.

During my graduate studies at Iowa State University, Dr. Kan Wang, Dr. Francois Torney, Lorena Moeller and I contributed an invited review to Current Opinions in Biotechnology entitled "Genetic engineering approaches to improve bioethanol production from maize" published June, 2007 (Torney *et al.* 2007). This review is reproduced herein as Chapter 5. My major contribution to this review is in the area of genetic engineering for the enhancement of abiotic stress tolerance. The thesis concludes with Chapter 6, a general overview of results and suggestions for further research.

1.1 Genetic Engineering and crop improvement

Current expectations of crop productivity demand stable yield over a wide range of soil and climate conditions. Stated generally, twenty-first century agriculture requires greater tolerance to stress. J.S. Boyer (1982) summarized stress as natural environments in which plants are prevented from expressing their full genetic potential for reproduction. Based on a comparison to record yields (an estimate of genetic potential), crops reach approximately 22% of that potential, with abiotic factors- temperature, soil chemistry and water availability- accounting for nearly 70% of yield loss (Boyer 1982).

Breeding has increased the record yields since the early twentieth century. Improvements in land management provide more favorable environments for plants to reach their genetic potential, however, economic and environmental considerations limit these strategies in the United States and the world, especially in developing countries (Boyer 1982; Pretty 2007). Selective breeding has shifted crop populations towards increased stress tolerance (Munns et al. 2006; Senthil-Kumar et al. 2007). Genetic engineering complements traditional breeding primarily through the benefits of decreased generation time and increased diversity of traits. Whereas traditional breeding may take several generations to introgress a desired trait into an agronomically fit line, genetic engineering can draw genes from any species and introduce those genes directly into a desired crop line.

Most commercialized transgenic crops in America express traits for either resistance to insect pests or tolerance to herbicide (Eizaguirre et al. 2006). The next generation of genetically engineered crops will be protected from abiotic stress. Abiotic stresses such as drought, salinity and cold are perceived differently by plants. However,

they do evoke similar molecular responses. Shared themes in stress physiology include osmotic stress, production of reactive oxygen species (ROS), and protection of proteins or refolding of denatured proteins (Seki et al. 2003; Wang et al. 2004; Zhang et al. 2004). Engineering for one stress may produce favorable effects on tolerance to other stresses due to these commonalities.

1.1.1 Common themes in plant response to abiotic stress

Osmotic stress

Plants encounter adverse osmotic conditions during drought stress, freezing or high temperature stresses or during saline stress (Seki *et al.* 2003; Munns 2005; Verslues *et al.* 2006). Drought limits water availability, whereas saline conditions hinder water uptake by osmotic effects. The early phases of physiological response to both saline conditions and drought stress are mediated by abscisic acid (ABA). Other signals from the root may augment ABA responses (Munns 2005). Freezing stress produces changes in cell osmotic conditions by drawing water out of the cytoplasm into the intercellular regions, often resulting in the formation of intercellular ice (Verslues *et al.* 2006). The control of gene transcription by ABA responsive promoters is common to cold and drought (Zhang *et al.* 2004). Small molecules thought to act as osmoprotectants are produced under both of these conditions (Hasegawa *et al.* 2000; Munns 2005; Vinocur and Altman 2005; Umezawa *et al.* 2006).

Reactive oxygen species (ROS)

ROS produced during stress serves both as secondary messengers and as components of stress-related damage (Xiong *et al.* 2002; del Rio *et al.* 2006; Kwak *et al.*

2006). ROS are produced as a result of normal metabolism but are also highly regulated, mediating programmed cell death (PCD) and pathogen defense (Mittler 2002; Rhoads *et al.* 2006; Torres *et al.* 2006; Van Breusegem and Dat 2006). Conditions found to induce ROS include drought, desiccation, salt, chilling, heat shock, heavy metals, ultraviolet radiation and ozone (Mittler 2002; Pitzschke and Hirt 2006). ROS produced during stress are generated through photorespiration, the two photosystems and mitochondrial respiration, as in normal plant metabolism. However, under conditions of severe stress, ROS levels accumulated in the cell exceed the steady state (Mittler 2002; Halliwell 2006)

Cellular damage and death due to excessive ROS can occur through multiple causes: cellular disruption due to membrane lipid peroxidation, loss of metabolic function following protein oxidation or enzyme inhibition, damage to nucleic acids, or the triggering of PCD (Halliwell 2006; Shulaev and Oliver 2006). Mechanisms of scavenging and detoxification of ROS restore balance to the cell after the stress has passed; increasing the ability of cells to detoxify or scavenge ROS may prolong the survival of the tissue during encounters with abiotic stress.

Protein protection and folding

Heat shock proteins and molecular chaperones are called into play under stress conditions. Members of the two best-studied families of such proteins, the Hsp70 family and the chaperonins, are inducible by stress conditions other than heat. The Hsp family and chaperonins help maintain cellular homeostasis by refolding proteins, preventing their denaturation, or preventing the formation of protein aggregates (Wang *et al.* 2004). The low-molecular-mass Hsps (sHsps), are most prevalent in plants and respond to heat, drought, cold, salinity and oxidative stress (Sun *et al.* 2002). Compatible solutes may

behave as "chemical chaperones" during osmotic stress (Singer and Lindquist 1998; Diamant *et al.* 2001). Late-embryogenesis abundant (LEA) proteins also have proposed chaperone qualities (Goyal *et al.* 2005; Umezawa *et al.* 2006).

1.1.2 Genetic engineering strategies for increasing tolerance to abiotic stress

Numerous published reviews address the use of transgenic methods to enhance stress tolerance (Seki et al. 2003; Zhang et al. 2004; Bajaj and Mohanty 2005; Munns 2005; Vinocur and Altman 2005; Umezawa et al. 2006). Selected examples relating to osmotic stress, ROS detoxification and scavenging, and protein protection and folding are summarized below.

Osmotic stress

Osmotic stress accompanies drought stress, but is also caused by high saline, heat, cold and freezing stresses. An increase in metabolites known as compatible solutes or osmoprotectants (such as trehalose, glycine betaine and proline) occurs in response to osmotic stress. Much work in manipulating sugar alcohol, polyamine and amino acid synthesis has taken place in rice, tobacco, tomato and wheat (Abebe *et al.* 2003; Capell *et al.* 2004; Bajaj and Mohanty 2005; Vinocur and Altman 2005). Increasing trehalose accumulation in rice through the expression of a synthetic trehalose-6-phosphate synthase/phosphatase improved root and shoot growth under selected abiotic stress conditions and lessened damage to leaves and photosynthetic capacity following exposure to drought, salt and cold (Garg *et al.* 2002; Jang *et al.* 2003). Similarly, insertion of bacterial choline oxidase, *codA* from *Arthrobacter globiformis* into rice increased accumulation of glycine betaine in a crop which does not normally accumulate

this compatible solute (Wang *et al.* 2003). Glycine betaine accumulation offered some degree of protection against photosystem damage under 100mM salt and low temperature (5^{o}C) in the moderately salt tolerant japonica rice cultivar Nipponbare (Sakamoto *et al.* 1998). *A. globiformis codA* expressed in a salt-sensitive indica cultivar Pusa Basmati permitted greater survival of transgenic plants following one week of treatment with 150mM NaCl. Greater than 50% of transgenic plants exposed to salt stress were able to recover when the stress was removed, whereas non-transgenic control plants died during the recovery period (Mohanty *et al.* 2002). Expression of *Delta(1)-pyrroline-5-carboxylate synthetase* (*P5CS)* either from *Arabidopsis thaliana* or *Vigna aconitifolia* increased proline accumulation in tobacco. Plants expressing *P5CS* showed 100% survival following exposure to below-freezing temperatures for 24 hours; the treatment produced severe damage and leaf-soaking in non-transgenic plants (Parvanova *et al.* 2004).

Reactive oxygen species (ROS)

Oxidative stress caused by the accumulation of ROS is a common byproduct of exposure to severe environmental stress. Plants are capable of converting $O^{2\bullet-}$ to O_2 and H_2O_2 through the action of superoxide dismutases (SODs) and further remove H_2O_2 by catalases, peroxidases, and scavenger molecules such as ascorbate (Halliwell 2006). Overexpression of *MnSOD*s in maize protoplasts led to early successes in increasing tolerance to oxidative damage (Van Breusegem *et al.* 1999a). Expression of *FeSOD* from *Arabidopsis* in maize decreased leaf damage due to oxidative stress imposed by paraquat (Van Breusegem *et al.* 1999b). More recently, ascorbate peroxidase was

overexpressed in *Arabidopsis* producing transgenic plants that suffered less damage due to paraquat treatment as measured by chlorophyll loss (Murgia *et al.* 2004).

Protein protection and folding

Heat shock proteins may function as stabilizing agents during stress. Rice expressing high levels of Hsp101 and Hsp17.7 proteins is more tolerant to high temperature stress (Bajaj and Mohanty 2005). Dehydrins and late embrogenesis abundant (LEA) proteins may also behave as chaperones, protecting proteins from denaturing or aiding in proper folding under stress conditions (Wang *et al.* 2004; Vinocur and Altman 2005). Expression of barley LEA protein gene *HVA1* in rice delayed leaf wilting under drought and salt-stress, and *HVA1* transgenic rice seedlings maintained their growth rate during the first five days of salt stress (Xu *et al.* 1996).

Genes involved in stress response fall into two categories: functional proteins, whose properties directly influence structural or metabolic events in the cell, and signal transduction components, including kinases, secondary messengers and transcription factors. The examples cited above represent the use of functional proteins as transgenes. Functional proteins are considered the front line defense against stress, and not surprisingly are a common means of genetic manipulation to enhance tolerance to cold, drought and salinity as well as to combinations of these stresses. More recent approaches to engineer stress tolerance make use of transcriptional control and associated signaling components.

Transcription factors

Transcriptional control over multiple genes is initiated by the binding of transcription factors to 5' elements. Overexpression of various classes of stress-related

transcription factors increase the expression of stress-related genes and can increase the survivability of transgenic organisms. ABA-responsive transcription factors such as ABF families of transcription factors are capable of triggering tolerance to multiple stresses when overexpressed (Seki *et al.* 2003). Overexpression of an *Arabidopsis* bZIP protein ABF3 in *Arabidopsis* enhanced tolerance to low and high temperatures (2^{o}C, 48^{o}C respectively) and general oxidative stress as demonstrated by Kim *et al.* (2004). *Arabidopsis* overexpressing a constitutively active mutant form of *Arabidopsis* ABF2 displayed enhanced drought tolerance with near 100% survival after 12d of water withholding; transgenic plants also had increased production of LEA proteins (Fujita *et al.* 2005). Overexpression of other members of the bZIP and MYB/MYC family of transcription factors has also been used to produce drought tolerant *Arabidopsis* and rice transgenic lines (Zhang *et al.* 2004; Umezawa *et al.* 2006). Much attention is paid to the AP2/ERF family and the DREB1/CBF transcription factors. First demonstrated as a potential method of enhancing stress tolerance in 1998 (Jaglo-Ottosen *et al.* 1998), *Arabidopsis CBF1* has been used to improve tolerance to multiple stresses in *Brassica napus*, tomato, strawberry, wheat, tobacco and rice (reviewed in (Zhang *et al.* 2004). Likewise, *Arabidopsis CBF3* has been tested and shown to improved plant growth and survivability under drought stress (reviewed in Umezawa *et al.* 2006).

Signaling components

Protein kinases, including the mitogen-activated protein kinases (MAPKs), are widely associated with response to biotic and abiotic stress (Asai et al. 2002; Jonak et al. 2004; Boudsocq and Lauriere 2005; Nakagami et al. 2005). Overexpression of *OsCDPK7*, encoding a calcium-dependent protein kinase, generated rice better able to

recover from cold stress (48h at 4^{o}C) as determined by chlorophyll fluorescence measurements. *OsCDPK7* transgenic plants also displayed delayed wilting under salt and drought conditions (Saijo *et al.* 2000).

MAPKs act within a pathway of sequential phosphorylation referred to as a cascade (see Figure 1.1). At each level, different designations are given for each type of kinase. MAP3Ks are closest to the perception of stress, and by definition, phosphorylate MAP2Ks. MAP2Ks are responsible for the phosphorylation of MAPKs. The substrates of the MAPKs are often transcription factors, but may include functional proteins activated or deactivated by phosphorylation (Ichimura et al. 2002).

Studies have made use of MAPK cascade element overexpression as both endogenous genes (Kovtun *et al.* 2000; Zhang and Liu 2001; Asai *et al.* 2002; Xiong and Yang 2003; Teige *et al.* 2004) and exogenous genes (Kovtun *et al.* 1998; Ren *et al.* 2002; Cheong *et al.* 2003; Lee *et al.* 2004). In many cases, these experiments were meant to study signaling interactions and developmental regulation. One example was the use of a gain of function mutant of YDA (an *Arabidopsis* MAP3K) to study stomatal development (Wang *et al.* 2007). Another example was array experiments making use of *Arabidopsis* overexpressing *AtMKK2* (Teige *et al.* 2004).

Manipulations of MAPKs reveal the parts they may play in pathogen response. Overexpression of tobacco *SIPK* increased hypersensitive response (HR) in leaves (Zhang and Liu 2001). Later work revealed that both overexpression and RNAi of *SIPK* produced necrosis in transgenic tobacco at levels of oxidative stress that did not affect wild type controls (atmospheric exposure to 500 parts per billion ozone) (Samuel and Ellis 2002). The *Arabidopsis* MAP2Ks *AtMEK4* and *AtMEK5* were expressed under an

inducible promoter; transcription stimulated by application of the inducer led to HR like cell death and H_2O_2 production (Ren *et al.* 2002).

Specific uses of MAPKs as a stress-tolerance engineering strategy include the overexpression of rice *BWMK1* in tobacco, which produced a lesion mimic phenotype in the transgenic plants. The transgenics also had increased abundance of pathogenesis-related (PR) gene transcripts and remained symptom free as many as eight days following infection with *Phytophthora parasitica* var nicotianae (Cheong et al. 2003). A similar increase in biotic stress tolerance was obtained by the expression of *MK1* from *Capsium annum* in rice, which increased expression of *PR* genes as well as levels of jasmonic acid and contributed to increased tolerance to rice blast infection (Lee et al. 2004). Transcriptional as well as physiological effects of overexpression of a MAP2K, *AtMKK2* in *Arabidopsis*, include increased transcripts of stress-related genes and enhanced tolerance to freezing and salt (Teige et al. 2004). However, the use of MAP3Ks as an engineering strategy and the effects of expressing MAP3Ks as exogenous transgenes in whole plants are only beginning to be investigated. A model for this approach has been the expression of the tobacco MAP3K, *Nicotiana* Protein Kinase 1 (Kovtun *et al.* 1998; Shou *et al.* 2004a,b).

1.2.2 *Nicotiana* Protein Kinase 1 (*NPK1*)

Nicotiana Protein Kinase 1 (*NPK1*) was first described in connection with cell division (Nakashima *et al.* 1998; Nishihama *et al.* 2002; Soyano *et al.* 2003). In tobacco, *NPK1* is expressed in actively dividing cells and not in mature tissue (Banno *et al.* 1993; Nakashima *et al.* 1998). Nishihama et al. (Nishihama *et al.* 2001) found *NPK1*

expression was highest during the mitotic stage of the cell cycle and had the highest proportion of phosphorylated NPK1 protein (active NPK1) during mitosis. Additionally, NPK1 localizes on the equatorial plane of the phragmoplast during telophase, helping direct the partitioning of daughter cells (Nishihama *et al.* 2001). Evidence for it being a MAP3K lies in its sequence similarity to ERK Kinase Kinase–like protein (Nakashima *et al.* 1998) of the serine/threonine kinase class, sequence homology with the yeast MAP3Ks STE11, BCK1 and Byr2, as well as the ability to functionally complement yeast MAP3K mutants lacking BCK1 activity (Banno *et al.* 1993).

In addition to its developmental role, NPK1 may potentially be necessary for innate immunity and auxin regulation. Virus-induced gene silencing of *NPK1* resulted in abnormal cellular phenotypes in tobacco, but also impaired disease resistance (Jin *et al.* 2002), indicating a potential role in stress response. Additionally, overexpression of the NPK1 kinase domain (constitutively active NPK1) in tobacco led to lines with low seed germination due to defects in embryo and endosperm development, potentially due to decrease of auxin-sensitve expression (Kovtun *et al.* 1998). This work also revealed that expression of *NPK1* under a constitutive auxin-insensitive promoter can activate MAPK proteins and also prevent activation of auxin-sensitive promoters in maize protoplasts (Kovtun *et al.* 1998).

In subsequent work, Kovtun et al (2000) demonstrated that tobacco expressing the constitutively active deletion mutant of *NPK1* (*caNPK1*) survived heat (48^{o}C) that resulted in 100% mortality of non-transgenic tobacco. Moreover, plants recovered and regrew faster following exposure to below freezing temperature, and had a higher survival rate following exposure to Murashige and Skoog (MS) media supplemented with

300mM salt. The mechanism by which tolerance is conferred was postulated to follow the model of constitutive expression of the *Arabidopsis NPK1* homolog, *ANP1*, in maize protoplasts, which led to the activation of H_2O_2-inducible promoters (Kovtun *et al.* 2000).

Expression of *caNPK1* as an exogenous transgene in a whole plant system was studied by Shou et al (2004a,b). They demonstrated that maintenance of apparent photosynthetic rate under drought conditions correlated with level of transgene expression; transgenic plants grown in drought conditions showed increased kernel number under drought and maintained kernel weight relative to wild type (Shou *et al.* 2004b). In addition, *caNPK1*-expressing maize subjected to cold survived up to three hours longer than their non-transgenic siblings at -5^{o}C during constant freezing; they were capable of withstanding below-freezing temperatures 1-2 degrees lower than the non-transgenic plants in graduated freezing tests (Shou *et al.* 2004a). Additionally, in the absence of stress the transgenic plants show upregulation of stress-related genes in an effect similar to short-term cold exposure (Shou *et al*. 2004a). Specifically, levels of transcript for the following genes were higher in *caNPK1* transgenic plants then their null segregants following exposure to 4^{o}C for 48 hours: *DREB1, EREBP, GPX2, HSP101, Jasmonic acid-inducible protein, P5CS, Pyruvate decarboxylase, CCR4-associated factor, GST1, HSP83*, and *proline transporter*. Interestingly, levels of *GST*, *HSP17.8*, and *PR1* mRNA levels were significantly increased over levels in non-transgenic plants in the absence of stress (Shou *et al.* 2004a). The stimulation of transcription in the absence of stress may be related to signaling through a substrate homolog, phosphorylation of MAP2Ks other than the substrate homolog (crosstalk), or the behavior of caNPK1 as a H_2O_2 signal mimic.

1.3 Purpose of the constitutively active mutant and this thesis

caNPK1 has the capacity to increase stress tolerance in plants when ectopically expressed (Kovtun *et al.* 2000; Shou *et al.* 2004a, b) and appears to influence expression of stress-related genes in the absence of stress (Shou *et al.* 2004a). The precise mechanism and impact of caNPK1 on kinase cascades has not yet been studied. In both tobacco and maize whole-plant systems expressing *caNPK1*, the effect on kinase gene expression or kinase activity was not examined. Additionally, studies on global transcriptional effects due to the expression of an exogenous MAP3K in plants have not previously been described.

The possibility that caNPK1 could activate homologs of its natural substrate, or MAP2Ks other than homologs to its natural substrate, could impact stress response by increasing or decreasing tolerance. caNPK1 expression could possibly affect other functions such as cell division, PCD, or auxin response.

Based upon the research conducted in maize by Shou et al. (2004a), our hypothesis is that caNPK1 is capable of signaling through stress-related kinase cascades in the absence of stress. It is not clear whether the increased recruitment of stress-responsive genes in maize can be attributed primarily to post-translational modification of kinase proteins already in the cell or if transcription of stress-related MAPKs is induced in response to constitutively active kinase signaling. Given the high potential for crosstalk, it is also of interest to monitor expression of genes related to developmental-associated kinase cascades. **Using the abundant genetic resources of rice we will assess the impact of foreign MAP3K expression on kinase gene expression and signaling in the host.** Understanding the effects of a constitutively

active MAP3K on host gene expression will aid researchers in generating transgenic MAP3Ks that give desired effects while minimizing undesired side effects.

1.4 Rice as model system

We desired to monitor differences in gene expression in multiple pathways between *caNPK1*-expressing and wild type plants. To this end, we generated *caNPK1*-expressing Nipponbare rice and selected one line carrying a single insertion of *caNPK1* at a high level of transgene expression to use in salt stress, microarray and quantitative Real-Time PCR studies. The study of kinase signaling in a grass species is better undertaken in rice than in maize due to the greater number of characterized MAPKs (Table 1.1). The inbred nature of the rice genotype is another important consideration in choosing this model. Transgenic maize lines generated by Shou *et al*.(2004a) were in Hi type II maize genotype, a hybrid amenable for genetic transformation. The hybrid background is undesirable for the analysis of stress related responses due to genetic segregation of endogenous genes in the hybrid. The inbred nature of rice cultivar Nipponbare is expected to make the analysis less complex. Research on rice is greatly facilitated by the availability of genetic data, microarrays and cDNA libraries. Lastly, work that may lead to the agronomic improvement of rice is never in vain as rice is the staple source of carbohydrates for an estimated 50% of the world's population and serves as a model species for the grasses (Goff *et al.* 2002; Yu *et al.* 2002).

References

Abebe, T., Guenzi, A. C., Martin, B. and Cushman, J. C. (2003). Tolerance of Mannitol-Accumulating Transgenic Wheat to Water Stress and Salinity. Plant Physiology **131**: 1748-1755.

Agrawal, G. K., Agrawal, S. K., Shibato, J., Iwahashi, H. and Rakwal, R. (2003a). Novel rice MAP kinases OsMSRMK3 and OsWJUMK1 involved in encountering diverse environmental stresses and developmental regulation. Biochemical and Biophysical Research Communications **300**: 775-783.

Agrawal, G. K., Iwahashi, H. and Rakwal, R. (2003b). Rice MAPKs. Biochemical and Biophysical Research Communications **302**: 171-180.

Agrawal, G. K., Rakwal, R. and Iwahashi, H. (2002). Isolation of novel rice (Oryza sativa L.) multiple stress responsive MAP kinase gene, *OsMSRMK2*, whose mRNA accumulates rapidly in response to environmental cues. Biochemical and Biophysical Research Communications **294**: 1009-1016.

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F. M. and Sheen, J. (2002). MAP Kinase signalling cascade in *Arabidopsis* innate immunity. Nature **415**: 977-983.

Bajaj, S. and Mohanty, A. (2005). Recent advances in rice biotechnology-towards genetically superior transgenic rice. Plant Biotechnology Journal **3**: 275-307.

Banno, H., Hirano, K., Nakamura, T., Irie, K., Nomoto, S., Matsumoto, K. and Machida, Y. (1993). *NPK1*, a Tobacco Gene That Encodes a Protein with a Domain Homologous to Yeast BCK1, STE11, and Byr2 Protein Kinases. Molecular and Cellular Biology **13**: 4745-4752.

Berberich, T., Sano, H. and Kusano, T. (1999). Involvement of a MAP kinase, ZmMPK5, in senescence and recovery from low-temperature stress in maize. Mol Gen Genet **262**: 534-542.

Boudsocq, M. and Lauriere, C. (2005). Osmotic Signaling in Plant. Multiple Pathways Mediated by Emerging Kinase Families. Plant Physiol. **138**: 1185-1194.

Boyer, J. S. (1982). Plant Productivity and Environment. Science **218**: 443-448.

Capell, T., Bassie, L. and Christou, P. (2004). Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Proc. Natl. Acad. Sci. USA **101**: 9909-9914.

Cheong, Y. H., Moon, B. C., Kim, J. K., Kim, C. Y., Kim, M. C., Kim, I. H., Park, C. Y., Kim, J. C., Park, B. O., Koo, S. C., Yoon, H. W., Chung, W. S., Lim, C. O., Lee, S. Y. and Cho, M. J. (2003). BWMK1, a Rice Mitogen-Activated Protein Kinase, Locates in the Nucleus and Mediates Pathogenesis-Related Gene Expression by Activation of a Transcription Factor. Plant Physiol. **132**: 1961-1972.

del Rio, L. A., Sandalio, L. M., Corpas, F. J., Palma, J. M. and Barroso, J. B. (2006). Reactive Oxygen Species and Reactive Nitrogen Species in Peroxisomes. Production, Scavenging, and Role in Cell Signaling. Plant Physiol. **141**: 330-335.

Diamant, S., Eliahu, N., Rosenthal, D. and Goloubinoff, P. (2001). Chemical Chaperones Regulate Molecular Chaperones in Vitro and in Cells under Combined Salt and Heat Stresses. The Journal of Biological Chemistry **276**: 39586-39591.

Fu, S.-F., Chou, W.-C., Huang, D.-D. and Huang, H.-J. (2002). Transcriptional Regulation of a Rice Mitogen-Activated Protein Kinase Gene, *OsMAPK4*, in Response to Environmental Stresses. Plant Cell Physiology **43**: 958-963.

Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M. M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2005). AREB1 Is a Transcription Activator of Novel ABRE-Dependent ABA Signaling That Enhances Drought Stress Tolerance in *Arabidopsis*. The Plant Cell **17**: 3470-3488.

Garg, A. K., Kim, J.-K., Owens, T. G., Ranwala, A. P., Choi, Y. D., Kochian, L. V. and Wu, R. J. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc. Natl. Acad. Sci. USA **99**: 15898-15903.

Goff, S. A., Ricke, D., Lan, T.-H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., Hadley, D., Hutchinson, D., Martin, C., Katagiri, F., Lange, B. M., Mougharner, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W.-L., Chen, L., Cooper, B., Park, S., Wood, T. C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zarkikh, A., Shen, R., Sahasrabudhe, S., Thomas, A., Cannings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R. M., Bhatnagar, S., Adey, N., Rubano, T., Tusneem, N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A. and Briggs, S. (2002). A Draft Sequence of the Rice Genome (*Oryza sativa* L. ssp. japonica). Science **296**: 92-100.

Goyal, K., Walton, L. J. and Runnacliffe, A. (2005). LEA proteins prevent protein aggregation due to water stress. Biochem J. **388**: 151-157.

Halliwell, B. (2006). Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. Plant Physiol. **141**: 312-322.

Hardin, S. C. and Wolniak, S. M. (2001). Expression of the mitogen-activated protein kinase kinase ZmMEK1 in the primary root of maize. Planta **213**: 916-926.

Hasegawa, P. M., Bressan, R. A., Zhu, J.-K. and Bohnert, H. J. (2000). Plant Cellular and Molecular Responses to High Salinity. Annual Review of Plant Physiology and Molecular Biology **51**: 463-499.

He, C., Fong, S. H. T., Yang, D. and Wang, G.-L. (1999). BWMK1, a Novel MAP Kinase Induced by Fungal Infection and Mechanical Wounding in Rice. Molecular Plant-Microbe Interactions **12**: 1064-1073.

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Kreis, M., Zhang, S., Hirt, H., Wilson, C., Heberle-Bors, E., Ellis, B. E., Morris, P. C., Innes, R. W., Ecker, J. R., Scheel, D., Klessig, D. F., Machida, Y., Mundy, J., Ohashi, Y. and Walker, J. C. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends in Plant Science **7**: 301-308.

Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O. and Thomashow, M. F. (1998). *Arabidopsis* CBF1 Overexpression Induces COR Genes and Enhances Freezing Tolerance. Science **280**: 104-106.

Jang, I.-C., Oh, S.-J., Seo, J.-S., Choi, W.-B., Song, S. I., Kim, C. H., Kim, Y. S., Seo, H.-S., Choi, Y. D., Nahm, B. H. and Kim, J.-K. (2003). Expression of a Bifunctional Fusion of the Escherichia coli Genes for Trehalose-6-Phosphate Synthase and Trehalose-6-Phosphate Phosphatase in Transgenic Rice Plants

Increases Trehalose Accumulation and Abiotic Stress Tolerance without Stunting Growth. Plant Physiol. **131**: 516-524.

Jin, H., Axtell, M. J., Dahlbeck, D., Ekwenna, O., Zhang, S., Staskawicz, B. and Baker, B. (2002). NPK1, an MEKK1-like Mitogen-Activated Protein Kinase Kinase Kinase, Regulates Innate Immunity and Development in Plants. Developmental Cell **3**: 291-297.

Jonak, C., Nakagami, H. and Hirt, H. (2004). Heavy Metal Stress. Activation of Distinct Mitogen-Activated Protein Kinase Pathways by Copper and Cadmium. Plant Physiology **136**: 3276-3283.

Kim, J.-A., Agrawal, G. K., Rakwal, R., Han, K.-S., Kim, K.-N., Yun, C.-H., Heu, S., Park, S.-Y., Lee, Y.-H. and Jwa, N.-S. (2003). Molecular cloning and mRNA expression analysis of a novel rice (Oryza sativa L.) MAPK kinase kinase, *OsEDR1*, and ortholog of *Arabidopsis AtEDR1*, reveal its role in defense/stress signalling pathways and development. Biochemical and Biophysical Research Communications **300**: 868-876.

Kim, J.-B., Kang, J.-Y. and Kim, S. Y. (2004). Over-expression of a transcription factor regulating ABA-responsive gene expression confers multiple stress tolerance. Plant Biotechnology Journal **2**: 459-466.

Kovtun, Y., Chiu, W.-L., Tena, G. and Sheen, J. (2000). From the Cover: Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. PNAS **97**: 2940-2945.

Kovtun, Y., Chiu, W.-L., Zeng, W. and Sheen, J. (1998). Supression of auxin signal transduction by a MAPK cascade in higher plants. Nature **395**: 716-720.

Kwak, J. M., Nguyen, V. and Schroeder, J. I. (2006). The Role of Reactive Oxygen Species in Hormonal Responses. Plant Physiol. **141**: 323-329.

Lalle, M., Visconti, S., Marra, M., Camoni, L., Velasco, R. and Aducci, P. (2005). ZmMPK6, a novel maize MAP kinase that interacts with 14-3-3 proteins. Plant Molecular Biology **59**: 713-722.

Lee, D.-E., Lee, I.-J., Han, O., Baik, M.-G., Han, S.-S. and Back, K. (2004). Pathogen Resistance of Transgenic Rice Plants Expressing Mitogen-activated Protein Kinase 1, MK1, from Capsicum annuum. Molecules and Cells **17**: 81-85.

Lieberherr, D., Thao, N. P., Nakashima, A., Umemura, K., Kawasaki, T. and Shimamoto, K. (2005). A Sphingolipid Elicitor-Inducible Mitogen-Activated Protien Kinase Is Regulated by the Small GTPase OsRac1 and Heterotrimeric G-Protein in Rice. Plant Physiol. **138**: 1644-1652.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science **7**: 405-410.

Mohanty, A., Kathuria, H., Sakamoto, A., Mohanty, P., Murata, N. and Tyagi, A. (2002). Transgenics of an elite indica rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. Theoretical and Applied Genetics **106**: 51-57.

Munns, R. (2005). Genes and salt tolerance: bringing them together. New Phytologist **167**: 645-663.

Munns, R., James, R. A. and Lauchli, A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. Journal of Experimental Botany **57**: 1025-1043.

Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carravieri, S. and Soave, C. (2004). *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. The Plant Journal **38**: 940-953.

Nakagami, H., Pitzschke, A. and Hirt, H. (2005). Emerging MAP kinase pathways in plant stress signalling. Trends in Plant Science **10**: 339-346.

Nakashima, M., Hirano, K., Nakashima, S., Banno, H., Nishihama, R. and Machida, Y. (1998). The Expression Pattern of the Gene for NPK1 Protein Kinase Related to Mitogen-Activated Protein Kinase Kinase Kinase (MAPKKK) in a Tobacco Plant: Correlation with Cell Proliferation. Plant Cell Physiol. **39**: 690-700.

Nishihama, R., Ishikawa, M., Araki, S., Soyano, T., Asada, T. and Machida, Y. (2001). The NPK1 mitogen-activated protein kinase kinase kinase is a regulator of cell-plate formation in plant cytokinesis. Genes & Development **15**: 352-363.

Nishihama, R., Soyano, T., Ishikawa, M., Araki, S., Tanaka, H., Asada, T., Irie, K., Ito, M., Terada, M., Banno, H., Yamazaki, Y. and Machida, Y. (2002). Expansion of the Cell Plate in Protein Cytokinesis Requires a Kinesin-like Protein/MAPKKK Complex. Cell **109**: 87-99.

Parvanova, D., Ivanov, S., Konstantinova, T., Karanov, E., Atanassov, A., Tsvetkov, T., Alexieva, V. and Djilianov, D. (2004). Transgenic tobacco plants accumulating osmolytes show reduced oxidative damage under freezing stress. Plant Physiology and Biochemistry **42**: 57-63.

Pitzschke, A. and Hirt, H. (2006). Mitogen-Activated Protein Kinases and Reactive Oxygen Species Signaling in Plants. Plant Physiol. **141**: 351-356.

Pretty, J. (2007). Agricultural sustainability: concepts, principles and evidence. Phil. Trans. R. Soc. Lond. **Epub ahead of print**.

Ren, D., Yang, H. and Zhang, S. (2002). Cell Death Mediated by MAPK Is Associated with Hydrogen Peroxide Production in *Arabidopsis*. TheJournal of Biological Chemistry **277**: 559-565.

Reyna, N. S. and Yang, Y. (2006). Molecular Analysis of the Rice MAP Kinase Gene Family in Relation to *Magnaporthe grisea* Infection. Molecular Plant-Microbe Interactions **19**: 530-540.

Rhoads, D. M., Umbach, A. L., Subbaiah, C. C. and Siedow, J. N. (2006). Mitochondrial Reactive Oxygen Species. Contribution to Oxidative Stress and Interorganellar Signaling. Plant Physiol. **141**: 357-366.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000). Over-expression of a single Ca2+-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. The Plant Journal **23**: 319-327.

Sakamoto, A., Murata, A. and Murata, N. (1998). Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. Plant Molecular Biology **38**: 1011-1019.

Samuel, M. A. and Ellis, B. E. (2002). Double Jeopardy: Both Overexpression and Suppression of a Redox-Activated Plant Mitogen-Activated Protein Kinase Render Tobacco Plants Ozone Sensitive. The Plant Cell **14**.

Seki, M., Kamei, A., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2003). Molecular responses to drought, salinity and frost: common and different paths for plant protection. Current Opinion in Biotechnology **14**: 194-199.

Senthil-Kumar, M., Kumar, G., Srikanthbabu, V. and Udayakumar, M. (2007). Assessment of variability in acquired thermotolerance: Potential option to study genotypic response and the relevance of stress genes. Journal of Plant Physiology **164**: 111-125.

Shou, H., Bordallo, P., Fan, J.-B., Yeakley, J. M., Bibikova, M., Sheen, J. and Wang, K. (2004a). Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc. Natl. Acad. Sci. USA **101**: 3298-3303.

Shou, H., Bordallo, P. and Wang, K. (2004b). Expression of the *Nicotiana* protein kinase (NPK1) enhanced drought tolerance in transgenic maize. Journal of Experimental Botany **55**: 1013-1019.

Shulaev, V. and Oliver, D. J. (2006). Metabolic and Proteomic Markers for Oxidative Stress. New Tools for Reactive Oxygen Species Research. Plant Physiol. **141**: 367-372.

Singer, M. A. and Lindquist, S. (1998). Multiple Effects of Trehalose on Protein Folding In Vitro an In Vivo. Molecular Cell **1**: 639-648.

Song, D., Chen, J., Song, F. and Zhang, Z. (2006). A Novel Rice MAPK Gene, *OsBIMK2*, is Involved in Disease-Resistance Responses. Plant Biol. **8**: 587-598.

Song, F. and Goodman, R. M. (2002). *OsBIMK1*, a rice MAP kinase gene involved in disease resistance responses. Planta **215**: 997-1005.

Soyano, T., Nishihama, R., Morikiyo, K., Ishikawa, M. and Machida, Y. (2003). NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes & Development **17**: 1055-1067.

Sun, W., Van Montagu, M. and Verbruggen, N. (2002). Small heat shock proteins and stress tolerance in plants. Biochemica et Biophysica Acta **1577**: 1-9.

Teige, M., Scheikl, E., Eulgem, T., Doczi, R., Ichimura, K., Shinozaki, K., Dangl, J. L. and Hirt, H. (2004). The MKK2 Pathway Mediates Cold and Salt Stress Signaling in *Arabidopsis*. Molecular Cell **15**: 141-152.

Torney, F., Moeller, L., Scarpa, A. and Wang, K. (2007). Genetic engineering approaches to improve bioethanol production from maize. Current Opinion in Biotechnology **18**: 193-199.

Torres, M. A., Jones, J. D. G. and Dangl, J. L. (2006). Reactive Oxygen Species Signaling in Response to Pathogens. Plant Physiol. **141**: 373-378.

Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006). Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Current Opinion in Biotechnology **17**: 113-122.

Van Breusegem, F. and Dat, J. F. (2006). Reactive Oxygen Species in Plant Cell Death. Plant Physiol. **141**: 384-390.

Van Breusegem, F., Slooten, L., Stassart, J.-M., Botterman, J., Moens, T., Van Montagu, M. and Inze, D. (1999a). Effects of overproduction of tobacco MnSOD in maize chloroplasts on foliar tolerance to cold and oxidative stress. Journal of Experimental Botany **50**: 71-78.

Van Breusegem, F., Slooten, L., Stassart, J.-M., Moens, T., Botterman, J., Van Montagu, M. and Inze, D. (1999b). Overproduction of *Arabidopsis thaliana* FeSOD Confers Oxidative Stress Tolerance to Transgenic Maize. Plant Cell Physiol. **40**: 515-523.

Verslues, P. E., Agrawal, M., Katiyar-Agrawal, S., Zhu, J. and Zhu, J.-K. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. The Plant Journal **45**: 523-539.

Vinocur, B. and Altman, A. (2005). Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Current Opinion in Biotechnology **16**: 123-132.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J. C. and Zhang, S. (2007). Stomatal Development and Patterning Are Regulated by Environmentally Responsive Mitogen-Activated Protein Kinases in *Arabidopsis*. The Plant Cell **19**: 63-73.

Wang, W., Vinocur, B. and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta **218**: 1-14.

Wang, W., Vinocur, B., Shoseyov, O. and Altman, A. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends in Plant Science **9**: 244-252.

Wen, J.-Q., Oono, K. and Imai, R. (2002). Two Novel Mitogen-Activated Protein Signaling Components, OsMEK1 and OsMAP1, Are Involved in a Moderate Low-Temperature Signaling Pathway in Rice. Plant Physiol. **129**: 1880-1891.

Xiong, L., Schumaker, K. S. and Zhu, J.-K. (2002). Cell Signaling during Cold, Drought, and Salt Stress. The Plant Cell **Supplement 2002**: S165-S183.

Xiong, L. and Yang, Y. (2003). Disease Resistance and Abiotic Stress Tolerace in Rice Are Inversely Modulated by an Abscisic Acid-Inducible Mitogen-Activated Protein Kinase. The Plant Cell **15**: 745-759.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.-H. D. and Wu, R. (1996). Expression of a Late Embryogenesis Abundant Protein Gene, *HVA1*, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice. Plant Physiol. **110**: 249-257.

Yeh, C.-M., Hsiao, L.-J. and Huang, H.-J. (2004). Cadmium Activates a Mitogen-Activated Protein Kinase Gene and MBP Kinases in Rice. Plant Cell Physiol. **45**: 1306-1312.

Yu, J., Hu, S., Wang, J., Wong, G. K.-S., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., Zhang, X., Cao, M., Liu, J., Sun, J., Tang, J., Chen, Y., Huang, X., Lin, W., Ye, C., Tong, W., Cong, L., Geng, J., Han, Y., Li, L., Li, W., Hu, G., Huang, X., Li, W., Li, J., Liu, Z., Li, L., Liu, J., Qi, Q., Liu, J., Li, L., Li, T., Wang, X., Lu, H., Wu, T., Zhu, M., Ni, P., Han, H., Dong, W., Ren, X., Feng, X., Cui, P., Li, X., Wang, H., Xu, X., Zhai, W., Xu, Z., Zhang, J., He, S., Zhang, J., Xu, J., Zhang, K., Zheng, X., Dong, J., Zeng, W., Tao, L., Ye, J., Tan, J., Ren, X., Chen, X., He, J., Liu, D., Tian, W., Tian, C., Xia, H., Bao, Q., Li, G., Gao, H., Cao, T., Wang,

J., Zhao, W., Li, P., Chen, W., Wang, X., Zhang, Y., Hu, J., Wang, J., Liu, S., Yang, J., Zhang, G., Xiong, Y., Li, Z., Mao, L., Zhou, C., Zhu, Z., Chen, R., Hao, B., Zheng, W., Chen, S., Guo, W., Li, G., Liu, S., Tao, M., Wang, J., Zhu, L., Yuan, L. and Yang, H. (2002). A Draft Sequence of the Rice Genome (*Oryza sativa* L. ssp. indica). Science **296**: 79-92.

Zhang, J. Z., Creelman, R. A. and Zhu, J.-K. (2004). From Laboratory to Field. Using Information from *Arabidopsis* to Engineer Salt, Cold, and Drought Tolerance in Crops. Plant Physiol. **135**: 615-621.

Zhang, S. and Liu, Y. (2001). Activation of Salicylic Acid-Induced Protein Kinase, a Mitogen-Activated Protein Kinase, Induces Multiple Defense Responses in Tobacco. The Plant Cell **13**: 1877-1889.

	Rice (Oryza sativa)		Maize (Zea Mays)		Wheat (Triticum aestivum)	
	Definition	Public ID	Definition	Public ID	Definition	Public ID
MAPK	OsMPK1[1]	AB183398	ZmMPK6[9]	AY425817	46 KD p38-MAPK[11]	
	OsMPK2	BAC99508	ZmMPK5[10]	AB016802	putative MAPK1a*	AY881102
	OsMPK3[2]	AAG40591	ZmMPK4[10]	AB016801	putative MAPK1b*	AY881103
	OsMPK4[3]	CAB61889	putative MAPK*	DQ295030	MAP kinase*	EF672267
	OsMPK5[4]	AF479883	osmotic stimulation MAPK*	AY521228		
	OsMPK6	NM_197522	similar to ZmMAPK4*	AY486160		
	OsMPK7	AK099472	similar to ZmMAPK5*	AY486159		
	OsMPK8[5]	AJ512643	similar to OsMAPK3*[a]	AY486158		
	OsMPK9	AAT44204				
	OsMPK10	NM_192924				
	OsMPK11	BAD69155				
	OsMPK12[6]	AF177392				
	OsMPK13[7]	AY524973				
	OsMPK14	AAS98446				
	OsMPK15	ABA92667				
	OsMPK16	NM_192298				
	OsMPK17	AAT39148				
	OsMAPK3[8a]	AJ314582				
	OsBWMK2*	AY588939				
MAP2K	OsMEK1[12b]	DQ837532	ZmMEK1[13b]	U83625		
MAP3K	OsEDR1[14]	AY167575				

Table 1.1 Characterized and putative MAPKs,MAP2Ks and MAP3Ks identified in rice (Oryza sativa), Maize (Zea mays), and Wheat (Triticum aestivum) summarized from NCBI database. Rice MAPKs are defined using the name assigned in Reyna and Yang (2006), other names for rice MAPKs are given in footnotes 1-7. Footnotes 8-19 are citations for MAPKs, MAP2Ks and MAP3Ks from the three species not described in Reyna and Yang (2006). Direct submissions of nucleotide or protein sequences for MAPKs from rice, maize and wheat are denoted by an asterisk and are more fully described by Definition, nucleotide ID, authors, and submission date in the footnote. Putative homologs between species are marked with superscript letters a and b.

[1]OsMAPK6 (Lieberherr *et al.* 2005) or OsSIPK (AJ535841); [2]OsMAP3; [3]OsMSRMK3 (AJ512642) (Agrawal *et al.* 2003a,b) or OsMAPK4 (AJ251330) (Yeh *et al.* 2004; Fu *et al*. 2002; Agrawal *et al*. 2003b); [4]OsMAPK2 (AJ250331) (Agrawal *et al*. 2003b; Yeh *et al*. 2004); OsMSRMK2 (AJ486975) (Agrawal *et al*. 2002); OsMAP1 (AF216315) (Wen *et al.* 2002; Agrawal *et al*. 2003b); OsBIMK1 (AF332873) (Song and Goodman 2002; Agrawal *et al*. 2003b) or OsMAPK5 (Xiong and Yang 2003); [5]OsWJUMK1 (Agrawal *et al.* 2003a,b); [6]OsBWMK1 (Cheong *et al.* 2003; He *et al.* 1999; Agrawal *et al.* 2003a,b); [7]OsBIMK2 (Song *et al.* 2006); [8]Agrawal *et al.* 2003b, Yeh *et al.* 2004; [9]Lalle *et al*. 2005; [10]Berberich *et al.* 1999; [11]Wen *et al*. 2002; [12]Hardin and Wolniak 2001; [13]Kim *et al*. 2003.

*Direct submissions to NCBI as of October 29, 2007: (Definition, nucleotide ID, authors, submission date): **Rice:** (OsBWMK2, AY588939, Wang *et al*., Apr. 2, 2004). **Maize:** (putative MAPK, DQ295030, Zong *et al*., Nov. 16, 2005; osmotic stimulation MAPK, AY521228, Gu and Li, Mar.2, 2005; similar to ZmMAPK4, AY486160, Wang *et al*., Nov. 21, 2003; similar to ZmMAPK5, AY486159, Wang *et al*., Nov. 21, 2003; similar to OsMAPK3, AY486158, Wang *et al*., Nov. 21, 2003). **Wheat:** (putative MAPK1a, AY881102, Xu and Ma, Jan 8, 2005; putative MAPK1b, AY881103, Xu and Ma, Jan 8,2005; MAP kinase, EF672267, Vijayan *et al*., June 12, 2007).

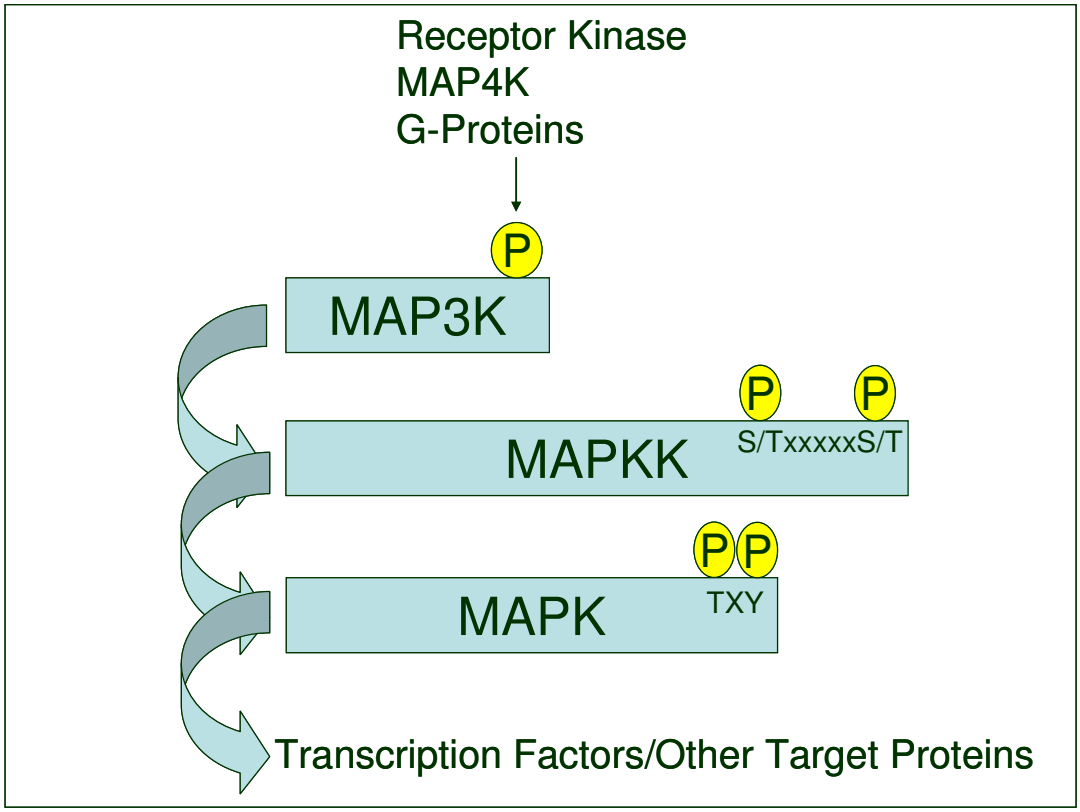

Figure 1.1 Mitogen-activated protein kinase cascade. The kinase that initiates the cascade, the MAP3K, activates the MAP2K through phosphorylation of serine or threonine residues. The MAP2K activates the MAPK by dual phosphorylation of both threonine and tyrosine residues (Ichimura *et al.* 2002). Activated MAPKs phosphorylate target proteins, often transcription factors.

Chapter 2: Generation and characterization of rice expressing constitutively active *Nicotiana* Protein Kinase 1 catalytic domain (*caNPK1*)

Abstract

A constitutively active form of the tobacco MAP3K Nicotiana protein kinase 1 catalytic domain (caNPK1) has the capacity to increase stress tolerance in plants where it is ectopically expressed (Kovtun *et al.* 2000; Shou *et al.* 2004a,b). To investigate the impact of *caNPK1* expression on stress tolerance and gene expression, we have generated *caNPK1* transgenic rice through *Agrobacterium*-mediated transformation. Twenty-five lines were generated, fifteen of which were analyzed for transgene copy number using Southern blot analysis. Two of fifteen lines (13% of lines analyzed) contained a single copy of the caNPK1 T-DNA cassette. All lines analyzed had low to medium copy numbers (60% 1-3 copies, 40% 4-5 copies). No more than five copies were found in any of the lines analyzed. Twelve of those lines characterized for copy number have transgene expression level assessed by Northern blot analysis and Reverse-Transcriptase PCR (RT-PCR). Trangene expression level as determined by Northern blot analysis correlated well with detection of *caNPK1* transcript by RT-PCR. RT-PCR was more sensitive for revealing low levels of transgene expression. Copy number and gene expression level in leaves do not appear to correlate inversely, suggesting that *Agrobacterium*-mediated transformation of rice callus with the *caNPK1* construct

produced a variety of unique transgenic events with a range of gene expression in mature leaves.

2.1 Introduction

Mitogen-activated protein kinases (MAPKs) mediate a diverse array of signals within the cell during responses to pathogen and abiotic stresses (Jonak et al. 2004; Nakagami et al. 2005). MAPK kinase kinases (MAP3Ks) function at the top of a kinase cascade, potentially leading to the activation of multiple transcription factors and stress-responsive genes. Thus more target genes could be activated through the MAP3K than by the overexpression of a single MAPK or single transcription factor alone. Therefore, an attractive strategy for maximizing the impact of genetic engineering using a single gene is to overexpress or express a constitutively active form of a MAP3K.

Kovtun et al (2000) demonstrated that tobacco plants expressing constitutively active *Nicotiana* Protein Kinase 1 (*caNPK1*), a MAP3K, survived heat (48^{o}C), tolerated below freezing temperature and had a higher survival rate following exposure to 300 mM salt than nontransgenic tobacco. When expressed as a transgene in maize, *caNPK1* conferred tolerance to drought conditions, where maintenance of apparent photosynthetic rate correlated with level of transgene expression. Encouragingly, transgenic maize showed increased kernel number under drought as well as a significantly higher kernel weight than wild type (Shou *et al.* 2004b). In cold tolerance studies, *caNPK1*-expressing maize survived up to three hours longer than their non-transgenic siblings at -5^{o}C under constant freezing; they were capable of withstanding below-freezing temperatures 1-2 degrees lower than the non-transgenic plants in graduated freezing tests (Shou *et al.*

2004a). Transcript levels of numerous stress-related genes were monitored in *caNPK1* trangenic maize and the null segregants in the absence of stress. mRNA levels of stress responsive protein genes such as *glutathione s-transferase* (*GST*), *heat shock protein 17.8* (*HSP17.8*), and *pathogenesis-related protein 1* (*PR1*) were significantly increased over levels found in non-transgenic plants. Such genes are also found to be upregulated in non-transgenic plants during short-term cold exposure. Following exposure to 4^{o}C for 48 hours, greater levels of transcripts were observed for stress-induced transcription factors *DREB1* (*drought-responsive element binding factor 1*) and *EREBP* (*ethylene-responsive element binding protein*), as well as *glutathione peroxidase 2* (*GPX2*), *Jasmonic acid-inducible protein*, *Pyruvate decarboxylase, CCR4-associated factor, glutathione s-transferase 1* (*GST1*), *heat shock proteins HSP101* and *HSP83*, *pyrroline-5-carboxylate synthetase* (*P5CS*) and a proline transporter in caNPK1 transgenic plants. (Shou *et al.* 2004a).

We have chosen to continue the examination of the impact of *caNPK1* expression on stress tolerance and gene expression using rice (*Oryza sativa* cv. Nipponbare). The precise mechanism and impact of *caNPK1* on stress-independent kinase cascades and has not yet been studied. In both tobacco and maize whole-plant systems expressing caNPK1, the effect on kinase gene expression or kinase activity was not examined. Rice is becoming a model system for cereal crops on par with *Arabidopsis*. The availability of whole genome arrays, genome annotation projects and the description of upwards of 16 characterized or putative MAPKs (Reyna and Yang 2005; Liu and Xue 2007) provides rich resources for transcriptome analysis. The maize genotype used in the studies of Shou et al., Hi II, is a hybrid with a mixed background of inbred A188 and B73

(Armstrong et al. 1991). Parental traits contained within Hi II hybrids segregate differently in each generation following self-fertilization. This complicates the separation of traits such as height and fertility and may potentially alter stress tolerance due to transgene effects. Nipponbare rice is ideal to study the effect of a single gene upon growth, performance under stress and gene expression because it is an inbred. Plants carrying a single copy of a transgene can be compared to nontransgenic Nipponbare in the same manner as near-isogenic lines.

To increase the likelihood of obtaining single-insertion transgenic events, Nipponbare callus was infected with *Agrobacterium* housing the *caNPK1* gene driven by a modified 35S promoter (Sheen 1993). Rice generated by transformation with *caNPK1* was analyzed for insertion number and gene expression. A high expressing single-insertion *caNPK1* transgenic line was selected for physiological assays under salt stress. Global gene expression using microarrays as well as expression of 17 specific MAPKs were compared between this transgenic line and Nipponbare plants in the absence of stress.

2.2 Materials and Methods

2.2.1 DNA construct and rice transformation

DNA construct pSHX004 (Figure 2.1a) carrying modified 35S promoter-caNPK1 was published previously (Shou et al., 2004b). The construct maintained the auxin-insensitive 35S promoter previously used by Kovtun et al. (1998; 2000) and originally described by Sheen (1993) (Figure 2.1b). The coding domain consists of the NPK1 catalytic region (aa 68-347) and lacks the c-terminal auto-regulatory domain (Figure

2.1c). The sequence of the caNPK1 cassette and primers used to verify the sequence of the insert are given in Appendix A.

The construct pSHX004 was introduced into *Agrobacterium* strain EHA101 and used to infect Nipponbare rice callus by the Iowa State University Plant Transformation Facility (protocol available through http://www.agron.iastate.edu/ptf/protocol/Rice.PDF).

2.2.2 Plant growth conditions

Soil culture

Initial work with the R_0 and R_1 plants used plants grown in soil. Controlled conditions of 12 hours light/12 hours dark and temperatures of 28^{o}C during the light period and 25^{o}C during the dark period were kept within a growth chamber (Conviron, USA). Seedlings in small pots were watered daily and transplanted to large pots containing slow release fertilizer (Osmocote 16-8-12) 10-12 days after sowing. After transplant, plants in large pots received water once daily with distilled water and were fertilized (Miracle-Gro 15-5-15) weekly followed 48-72 hours later by the addition of powdered iron (Ironite, Hummert).

Hydroponic culture

R_1 seeds were pregerminated by soaking in distilled water for 48 hours, then germinated on water-moistened paper towels in the dark at room temperature for 5 days. Germinated seedlings were transferred from the germination plate to hydroponic flats. An aquarium filter equipped with two rubber hoses supplied aeration for the roots. Media recipe for hydroponic growth of rice was kindly provided by Huixia Shou (Zhejiang University, China). Fresh nutrient solution (Table 2.1) was prepared weekly

and allowed to warm to 28°C within the growth chamber prior to transfer of flats to new solution. Growth chamber (Conviron, USA) conditions for rice were 12 hours light/ 12 hours dark at average temperatures of 28°C during light and 25°C during dark. Light intensity was ~300 μE/m^2s at mid-canopy height.

2.2.3 DNA extraction for polymerase chain reaction (PCR) and Southern blot analysis

DNA isolation for PCR

Leaf samples were collected from young leaves by enclosing a one-inch portion of the leaf tips within a 1.5 ml eppendorf tube and closing the lid. Tubes containing leaf samples were kept on ice after collection and homogenized with 600 μl DNA extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and ground well with autoclaved Kimble pestles. The homogenized sample underwent phenol and chloroform : isoamyl alcohol (24:1) extractions followed by isopropanol precipitation and ethanol wash. The dried pellet was resuspended in 50 μl TE 10/0.1 + 2 μl RNAse A.

Genomic DNA for Southern blot analysis

Tissue weighing 300 μg was collected from mature rice leaves (20-30 days after transplant) and was snap frozen in liquid nitrogen. Leaf tissue was stored at -80°C until processing. Leaves were ground to a fine powder in ceramic mortars with ceramic pestles (Coors, USA) with constant addition of liquid nitrogen. The ground tissue was mixed with 2X CTAB buffer (100 mM Tris-HCl pH 7.5, 700 mM NaCl, 10 mM EDTA, 1% CTAB, 1% Beta-mercapto-ethanol), followed by phenol and chloroform : isoamyl alcohol (24:1) extractions. Precipitation in isopropanol was followed by washing the

pellet in 1 ml of 75% ethanol. DNA, resuspended in 50 µl TE 10/0.1, was quantified by spectrophotometer.

2.2.4 Polymerase chain reaction (PCR)

Optimum conditions for the detection of *bar* and *caNPK1* genes differ and were determined separately. Primer sequences used for this analysis: NPK1int-f: 5'-TAA CAA ATG GAT GCT GAA GC-3'; NPK1int-r: 5'-CCA TCC CAA CAT AGT GAG AT-3'; Bar11: 5'-CAG CTG CCA GAA ACC CAC GT -3'; Bar12: 5'- CTG CAC CAT CGT CAA CCA CT-3'. The PCR master mixes and program conditions are listed in Table 2.2.

2.2.5 Southern blot analysis

Ten micrograms of each genomic DNA sample was digested overnight with 0.1 Units *Nco I*/µg DNA or 0.2 Units *EcoR I*/µg DNA in a volume of 20 µl. The entire volume of the digested DNA samples were electrophoresed overnight on a 1% agarose/1xTAE gel at low voltage (average 22V). Following depurination (in 0.2 M HCl), denaturation (in 0.5 M NaOH, 1 M NaCl) and neutralization (in 0.5 M Tris-HCl, 3 M NaCl) the gel was arranged for capillary blotting onto nylon membrane (Biorad). Nucleic acid was crosslinked using a UV crosslinker (Stratalinker) and incubated in 10 ml prehybridization solution (400 µM NaPO4, 6X SSC, 1% SDS plus Denhardt's solution and herring sperm DNA) for 3 hours at 65^{o}C. The probe used to detect the caNPK1 fragment was the *EcoR I/EcoR I* fragment from pSHX004 (Figure 1b). Radiolabel in the form of alpha-P^{32} dCTP was incorporated into the probe with the Prime-It II Random Primer Labeling Kit (Stratagene). Following overnight

hybridization, the membrane was washed at least twice (first with 1X SSC, 0.25% SDS followed by 0.5X SSC, 0.25 SDS) for 20 minutes each at 65^{o}C. Signal from probes was detected by autoradiography film (Kodak).

2.2.6 RNA extraction

Leaf tissue was collected by cutting a mature leaf from the plant with sharp scissors. Leaves were weighed and cut to 100 mg and then snap frozen in liquid nitrogen. Samples were stored at -80^{o}C until processing for RNA extraction. Leaf samples were first ground to fine powder using ceramic mortars and pestles (Coors, USA), with the constant addition of liquid nitrogen. The powdered leaf samples were immediately added to 1 ml TRI reagent and separated from protein and DNA through chloroform extraction. RNA in the aqueous phase was precipitated in isopropanol and the pellet washed with 75% ethanol prepared with DEPC-treated water. The pellets were then air dried and resuspended in 50 µl DEPC-treated water. Samples were quantified by spectrophotometer and quality-checked by non-denaturing gel electrophoresis.

2.2.7 Northern blot analysis

The Northern blot gel was prepared as followed: 10 µg RNA sample was first dried by speedvac (Thermo Scientific), and resuspended in 27 µl of 1x sample buffer (1X MOPS running buffer, 0.04 mg/ml Ethidium Bromide, 50% Deionized Formamide, 18% Formaldehyde brought to volume with DEPC-treated water). The resuspended RNA was then mixed with 3 µl of 10x loading dye (final concentration: 1 mM EDTA, 0.01% Xylene cyanole, 50% glycerol prepared with DEPC-treated water). Samples were electrophoresed for 4 hours at 60V and then transferred to a membrane by capillary

blotting, UV crosslinked (Stratalinker). The RNA membrane was first prehybridized in 20 ml of prehybridization buffer (1 mM EDTA pH 8, 0.5 M Na_2HPO_4 pH 7.2, 7% SDS) for 3 hours at 65^{o}C. The *EcoRI/EcoRI* fragment of pSHX004 was ^{32}P labeled through random priming with the Prime-It II Random Primer Labeling Kit (Stratagene) and 50 µl of the labeling reaction was added to the hybridization bottle. Then the membrane was hybridized 16 hours in the same buffer. The membrane was washed twice (first with 1 mM EDTA pH 8.0, 40 mM Na_2HPO_4 pH 7.2, 5% SDS, then with 1 mM EDTA pH 8.0, 40 mM Na_2HPO_4 pH 7.2, 1% SDS) for 30 minutes each and exposed to film (Kodak) for 1, 2 and 5 days to obtain best resolution. The membrane was stripped and probed with 18S probe (from RT-PCR product using 18S ribosomal RNA primers) as an internal RNA standard control. Exposure of 30 min yielded best resolution for membranes probed with 18S probe.

2.2.8 Reverse Transcription-PCR (RT-PCR) protocol

RT-PCR reactions were set up according to the instructions for the Qiagen One-Step RT-PCR kit. Suitable amplification of 18S rRNA and NPK1 fragment was obtained with 250 ng of total RNA and 250 µM of each primer (15 µM final primer concentration). Primer sequences: NPK1 forward: 5'- CTC TCC CAT CCC AAA TAT C -3'; NPK1 reverse: 5'- GAG CCA CCA GGA ACA AAT -3'; 18S forward: 5'- ACG AAC AAC TGC GAA AGC -3'; 18S reverse: 5'- CGG CAT CGT TTA TGG TTG -3'. A "No RT" serving as a control for DNA contamination was set up identically to the NPK1 reaction, however, the tubes for "No RT" were not placed in the thermalcycler at the beginning of the program and instead were held on ice until the RT portion (50^{o}C) is

completed and the PCR portion of the program began. The annealing temperature used for the PCR portion was 56°C.

2.3 Results and Discussion

2.3.1 Generation of transgenic rice carrying the caNPK1 gene

A total of 105 bialaphos resistant rice lines were generated from 25 transgenic events. We obtained from the Iowa State University Plant Transformation Facility one R_0 plantlet per event, transplanted to soil and raised for seed. Seed yield varied greatly between lines (Table 2.3). R_0 plants from line 46.1, 48.1, and 61.1 yielded no seeds. Leaf tissue was collected from these R_0 plants for DNA analysis, but without seeds, these lines could not be used in further studies. Eleven lines produced eleven or less seeds. Leaf tissue from the R_0 plants of the low yielding lines 2.2, 6.1, 10.1, 49.1, 105.1 and 199.1 were collected, but not from 17.1, 19.1, 22.1, 122.1, or 192.1. Attempts to germinate and grow R_1 plants from these five lines exhausted the supply of seed. Whether the failure of these R_1 seedlings to germinate and thrive in soil was due to an effect of the caNPK1 gene upon embryo development (Kovtun *et al.* 1998) was not investigated. At the time, the failure of seedlings to grow in soil was prevalent in both transgenic and nontransgenic rice. Five R_1 or R_2 seedlings of several lines were grown in hydroponic culture (Figure 2.3) for tissue collection for DNA and RNA analysis and height measurements taken at 21 days after planting (Table 2.4). The majority of lines did not differ from the wild type in height, however, lines 2.2, 7.1, 9.1, 10.1, 21.1, 47.1, and 178.1 were significantly shorter than the wild type (Student's t, α=0.05), however,

these lines were not significantly different in height from their null segregants, when null segregants were among the group grown.

2.3.2 Transgene analysis of caNPK1 plants

Southern blot analysis was performed on R_0 plants by François Torney where leaf tissue could be obtained (Figure 2.3). Figure 2.4 shows additional lines characterized at the R_1 generation. Genomic DNA was digested with *EcoR I* to demonstrate the presence of at least one intact copy of the *caNPK1* expression cassette. *Nco I* cuts once within the T-DNA cassette and can be used to estimate copy number. Two of the fifteen lines carried a single copy of the transgene, seven held 2-3 copies and six had 4 or 5 copies. Transgene insertions at greater than five copies were not observed.

These results are consistent with previous *Agrobacterium*-mediated transformation using the pSHX004 plasmid. Shou et al. (2004c) produced 24 transgenic maize events carrying the *caNPK1* transgene. Transgene copy number was estimated both by Southern blot analysis and relative quantification through real-time PCR of genomic DNA. Maize transformed with *caNPK1* through *Agrobacterium* infection carried low copy numbers, with 22 of 24 events having 1-3 copies (92%) and 12% displaying a single copy;, similarly, of the fifteen rice lines surveyed, 60% carried 1-3 copies of the transgene and 13% were determined to have a single insertion.

2.3.3 Transcript analysis of NPK1 plants

Both Northern blot analysis and RT-PCR are semi-quantitative measures of gene expression. RT-PCR was shown to be a more sensitive method of detecting low expression (Table 2.3). Portions of one developed film from Northern blot analysis of R_1

lines are shown in Figure 2.5. Screening of NPK1 gene expression using Northern blot analysis indicates that lines displayed different levels of NPK1 transcript, similar to the observation made for transgenic maize plants (Shou et al, 2004a).

PCR screening for transgenics using primers within the NPK1 coding region detected the presence of the transgene without amplification of endogenous genes, allowing for accurate RT-PCR analysis of *caNPK1* gene expression (Figure 2.6). In samples where genomic contamination was absent, RT-PCR detection of the transgene is due to abundance of transcribed caNPK1 alone. In such samples, relative RT-PCR gene expression levels correlated well with levels of expression detected by Northern blot analysis.

2.4. Summary and Conclusions

We desire to monitor differences in gene expression in multiple pathways between caNPK1-expressing and wild type rice plants. To this end, we have generated caNPK1-expressing Nipponbare rice. We have selected the line 178.1, carrying a single insertion of caNPK1 at a high level of transgene expression, to use in salt stress, microarray and quantitative Real-Time PCR studies.

Of the 25 plants obtained from the Iowa State University Plant Transformation Facility, which represent 25 unique transgenic events, 15 were analyzed by Southern blot analysis. Two lines were found to have single insertions of the transgene, lines 178.1 and 6.1. Further, levels of transgene expression in 12 of those 15 lines were measured both by Northern blot analysis and RT-PCR. Line 6.1 could not be analyzed for transgene expression due to the death of the R_1 seedlings grown in soil. Line 178.1 was among four

lines determined to have strong and high expression as determined by Northern blot analysis and RT-PCR, respectively. Line 178.1 was selected for further work with microarray and physiology studies due primarily to the presence of a single insertion which facilitates comparison of transgenic and nontransgenic lines by reducing the genomic differences to a single locus.

Acknowledgements

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References

Armstrong, C. L., Green, C. E.,Phillips, R. L. (1991). "Development and availability of germplasm with high Type II culture formation response." Maize Genet. Coop. News letter **65**: 92-3.

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Kreis, M., Zhang, S., Hirt, H., Wilson, C., Heberle-Bors, E., Ellis, B. E., Morris, P. C., Innes, R. W., Ecker, J. R., Scheel, D., Klessig, D. F., Machida, Y., Mundy, J., Ohashi, Y. and Walker, J. C. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends in Plant Science **7**: 301-308.

Jonak, C., Nakagami, H. and Hirt, H. (2004). Heavy Metal Stress. Activation of Distinct Mitogen-Activated Protein Kinase Pathways by Copper and Cadmium. Plant Physiology **136**: 3276-3283.

Kovtun, Y., Chiu, W.-L., Tena, G. and Sheen, J. (2000). From the Cover: Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. PNAS **97**: 2940-2945.

Kovtun, Y., Chiu, W.-L., Zeng, W. and Sheen, J. (1998). Supression of auxin signal transduction by a MAPK cascade in higher plants. Nature **395**: 716-720.

Liu, Q. and Xue, Q. (2007). Computational identification and phylogenetic analysis of the MAPK gene family in *Oryza sativa*. Plant Physiology and Biochemistry **45**: 6-14.

Nakagami, H., Pitzschke, A. and Hirt, H. (2005). Emerging MAP kinase pathways in plant stress signalling. Trends in Plant Science **10**: 339-346.

Reyna, N. S. and Yang, Y. (2006). Molecular Analysis of the Rice MAP Kinase Gene Family in Relation to *Magnaporthe grisea* Infection. Molecular Plant-Microbe Interactions **19**: 530-540.

Sheen, J. (1993). Protein phosphatase activity is required for light-inducible gene expression in maize. The EMBO Journal **12**: 3497-3505.

Shou, H., Bordallo, P., Fan, J.-B., Yeakley, J. M., Bibikova, M., Sheen, J. and Wang, K. (2004a). Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc. Natl. Acad. Sci. USA **101**: 3298-3303.

Shou, H., Bordallo, P. and Wang, K. (2004b). Expression of the *Nicotiana* protein kinase (NPK1) enhanced drought tolerance in transgenic maize. Journal of Experimental Botany **55**: 1013-1019.

Shou, H., Frame, B. R., Whitham, S. A. and Wang, K. (2004c). Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. Molecular Breeding **13**: 201-208.

Element	ppm	mM
Nitrogen	40	2.86
Phosphorous	10	0.32
Potassium	40	1.02
Calcium	40	1.00
Magnesium	40	1.65
Manganese	0.5	0.0091
Molybdenum	0.05	0.00052
Boron	0.2	0.018
Zinc	0.01	0.00015
Copper	0.01	0.00016
Iron	2	0.036

Table 2.1. Composition of hydroponic solution. Nutrient content of growth solution given in parts per million (ppm) and millimolar (mM).

Master mix (per sample)	Bar	NPK1
Template	100ng	100ng
$MgCl_2$	1.5mM	3mM
Primers	0.2uM each	1uM each
Taq	0.04U	0.04U
Buffer	1X	1X
dNTPs	0.8mM	0.8mM
PCR Dye	1X	1X
Water	to 20ul	to 20 ul

Thermalcycler Program	MMP>Bar	PAT>NPK1B
Initial	95°C 5min	95°C 5min
Denature	95°C 45sec	95°C 45sec
Anneal	58°C 30sec	50°C 30sec
Extend	72°C 30sec	72°C 30sec
Repeat	x30	x40
Final	72°C 5min	72°C 5min

Table 2.2. PCR conditions for NPK1 and Bar gene detection.

Event	R_1 Seed	Copy Number	Expression By Northern	Expression By RT-PCR
2.2	6	4	No signal	Low
6.1*	5	1	ND	ND
7.1	29	2	Strong	High
9.1	100	4	Strong	High
10.1	2	5	Strong	High
12.1	179	3	Mid-no signal	Medium
16.2	24	5	No signal	Contam.
17.1*	3	ND	ND	ND
18.1	201	2	Mid strength	High
19.1*	2	ND	ND	ND
21.1	25	3	Strong	High
22.1*	11	ND	ND	ND
36.1	56	3	Mid strength	High
46.1**	0	ND	ND	ND
47.1*	56	4	ND	ND
48.1**	0	ND	ND	ND
49.1*	2	ND	ND	ND
51.1	57	2	Low	Medium
61.1*	0	ND	ND	ND
78.1	31	4	Low	Medium
105.1*	1	2	ND	ND
122.1*	4	ND	ND	ND
178.1	62	1	Strong	High
192.1*	3	ND	ND	ND
199.1*	3	ND	ND	ND

Table 2.3. Characterization of transgenics. Seed obtained from R_0 plants varied widely. Transgene copy number as determined by R_0 and R_1 Southern blot analysis, and caNPK1 gene expression as assessed by Northern blot analysis and RT-PCR detection.

*R_1 plants that were grown in soil and died before leaves could be collected for RNA.

		Height of 21 day old plants (mm)					
		Transgenic			Null Segregant		
Event	Relative Expression	Mean	SD	N	Mean**	SD	N
2.2	Low	332.67*	133.57	3	352.00	--	1
7.1	High	323.33*	30.24	3	334.00	--	1
9.1	High	311.60*	126.86	5	NA	NA	NA
10.1	High	267.00*	0.00	2	256.50	14.85	2
12.1	Medium	427.50	40.87	4	456.00	--	1
16.2		393.00	50.98	5	NA	NA	NA
18.1	High	370.67	26.76	3	398.00	11.31	2
21.1	High	335.25*	91.51	4	290.00	--	1
36.1	High	352.50	65.76	2	415.00	--	1
47.1		318.20*	50.98	5	NA	NA	NA
51.1	Medium	434.40	22.57	5	NA	NA	NA
78.1	Medium	376.63	27.56	3	391.00	21.21	2
178.1	High	336.80*	83.79	5	NA	NA	NA
Wild Type	None	NA	NA	NA	442.80	20.54	5

Table 2.4. Height data of transgenic lines grown in hydroponics. Measurements of plant height were taken on plants grown for seed increase. Five seeds of each line were planted and these measurements taken at 21 days after planting, when plants had developed the third leaf.

* Heights differed significantly from wild type (Student's t, a=0.05).

** In some lines only one plant was nontransgenic, in others no plants were shown to be nontransgenic segregants.

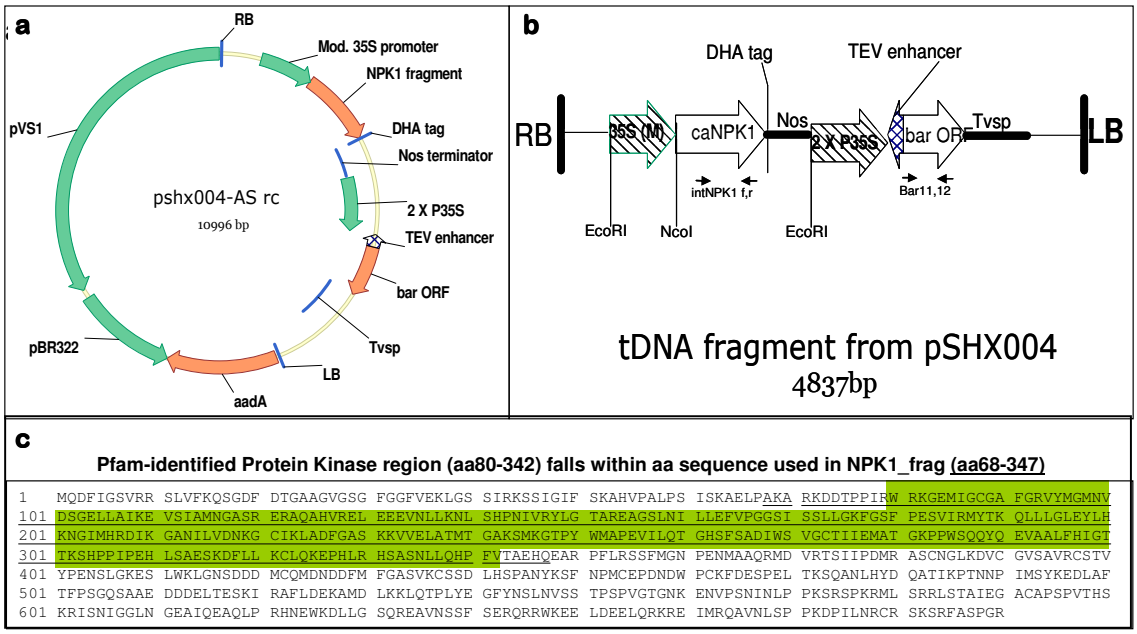

Figure 2.1. Construct used for transformation of Nipponbare rice. Plasmid map of pSHX004, a dual host vector (a). Map of tDNA fragment from pSHX004 showing 1.8kb EcoRI dropout and locations of primers for 604bp caNPK1 specific PCR product and 520bp Bar specific PCR product (b). Kinase domain (aa80-342) and caNPK1 coding domain translation (aa68-347) indicated on protein sequence of full-length tobacco NPK1 (BAA05648) (c).

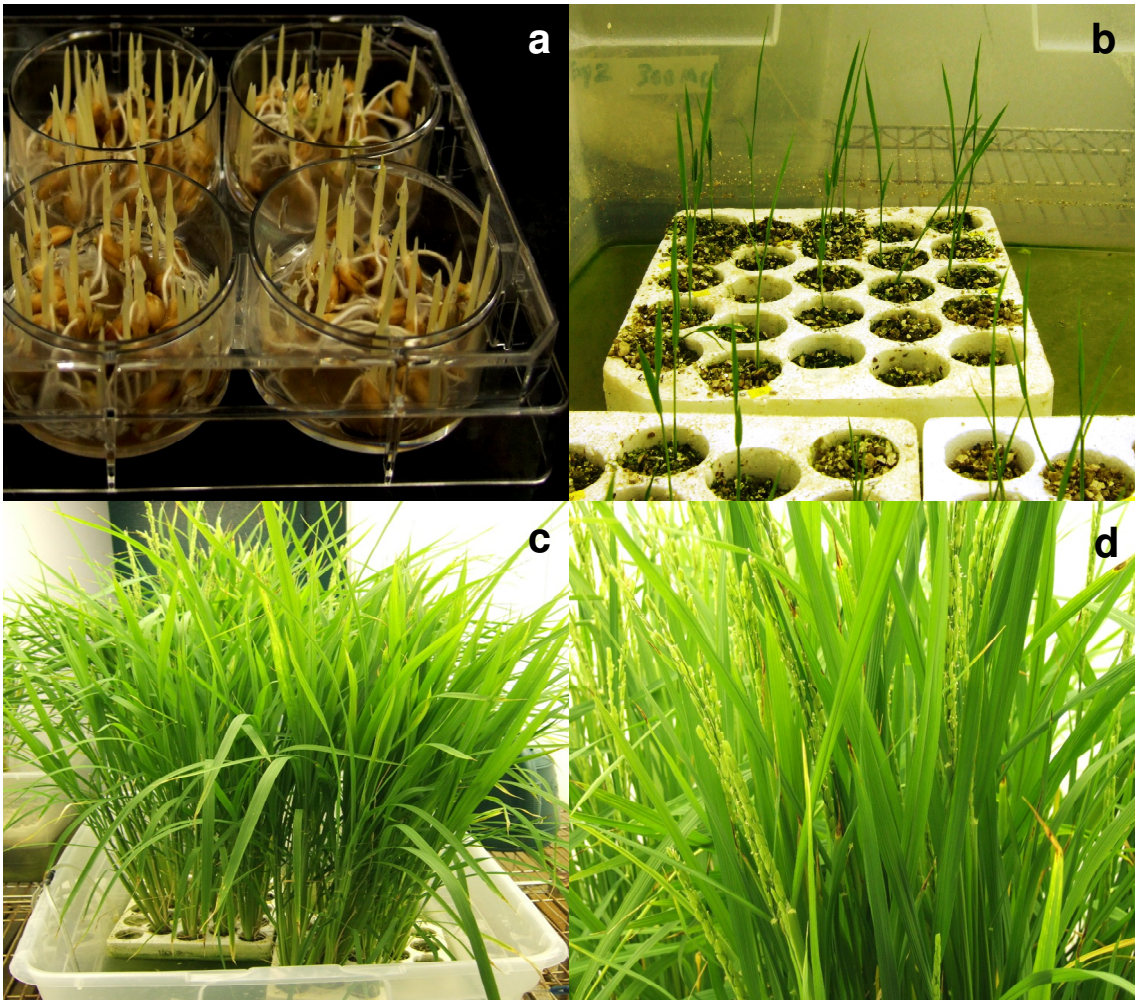

Figure 2.2. Rice hydroponic culture. Six-well cell culture plates layered with moistened paper towels serve as germination plates (a). Following two days of imbibition in distilled water and five days of germination in the dark at room temperature, germinated seedings are transferred to hydroponic flats. Seedlings at seven days after planting (b). Mature rice plants (28 days after planting) (c). Plants are capable of flowering and setting seed in the hydroponic system (flowers shown in panel d).

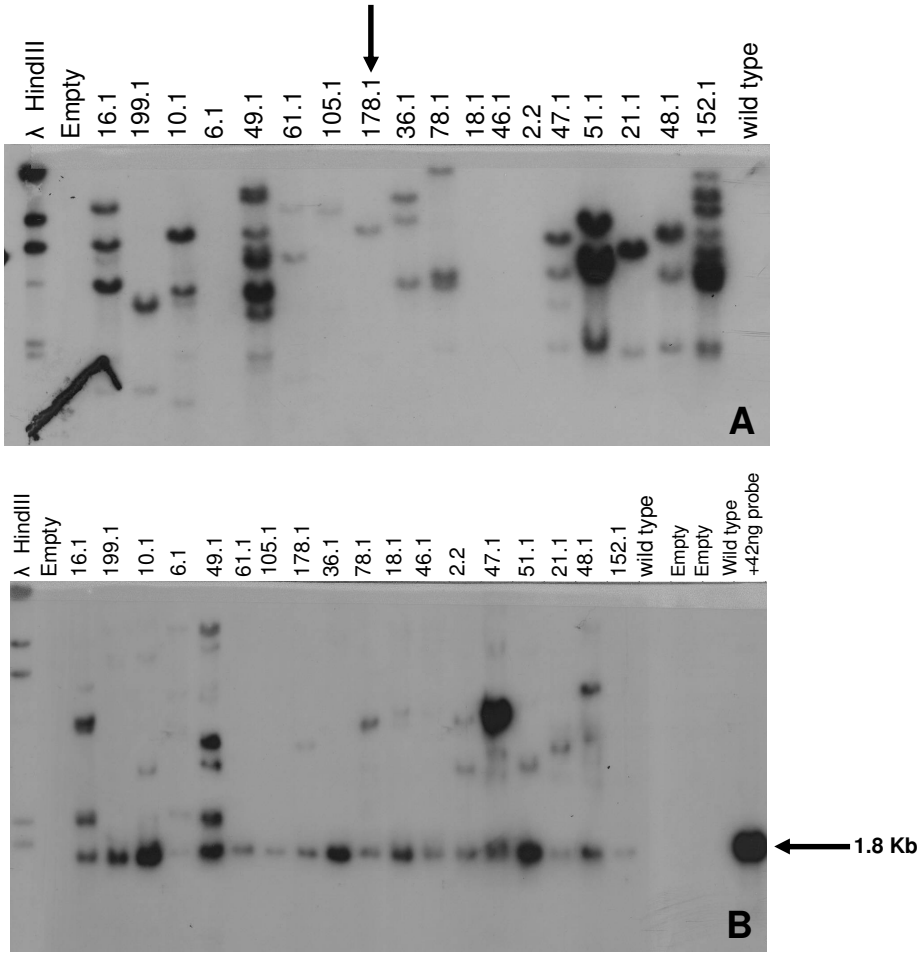

Figure 2.3. R_0 Southern blot as presented by F. Torney.
A: Rice plant genomic DNA was digested overnight by *NcoI* (one cut). The digestion products were separated on a 1% agarose gel and transferred using capillary method to a nylon membrane. DNA was crosslinked to the membrane using a UV crosslinker. The probe was labeled using a random priming kit (Qiagen) and alpha P^{32} dCTP. The purified probe was then purified using a G-50 column. Line 178.1 is indicated with a downward pointing arrow.
B: The exact same procedure as described in A was applied to *EcoRI* digested genomic DNA. *EcoRI* cuts twice and excises the NPK1 expression cassette. The 1.8kb EcoRI/EcoRI fragment corresponding to the EcoRI/EcoRI fragment of pSHX004 (see Figure 2.1 b).

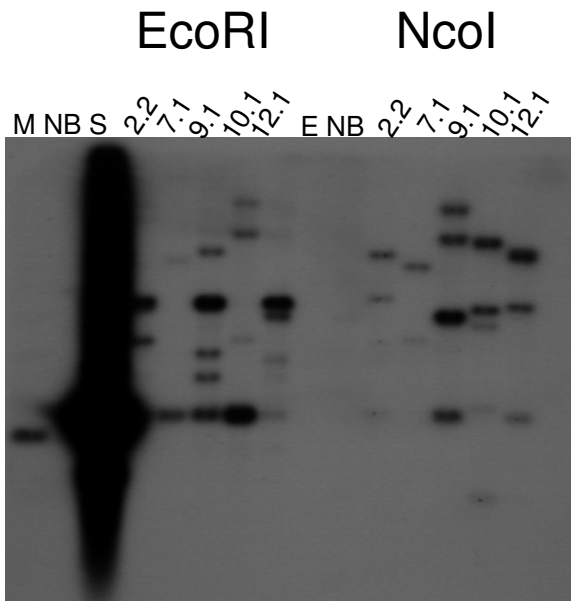

Figure 2.4. R_1 Southern blot of lines 2.2, 7.1, 9.1, 10.1, 12.1. 48 hour exposure of membrane probed with *EcoRI/EcoRI* fragment of pSHX004. Unlike the R_0 Southern blot analysis performed by François Torney, the probe was labeled using a Stratgene random priming kit and the probe was not column purified. The incorporated radiolabel was alpha-P^{32} dCTP. Invitrogen 1kb Track-it marker (M) contained a fragment that hybridized the probe. Genomic DNA from a Nipponbare (nontransgenic plant) served as negative control (NB) and Nipponbare genomic DNA spiked with 4.2ng of the *EcoRI/EcoRI* fragment of pSHX004 was intended to serve as a positive control, but was very intense following hybridization. The *EcoRI* fragment containing caNPK1 is visible in each R_1 line and copy numbers as estimated by *NcoI* fragments correspond well with those seen in the R_0 blot for lines appearing in both blots.

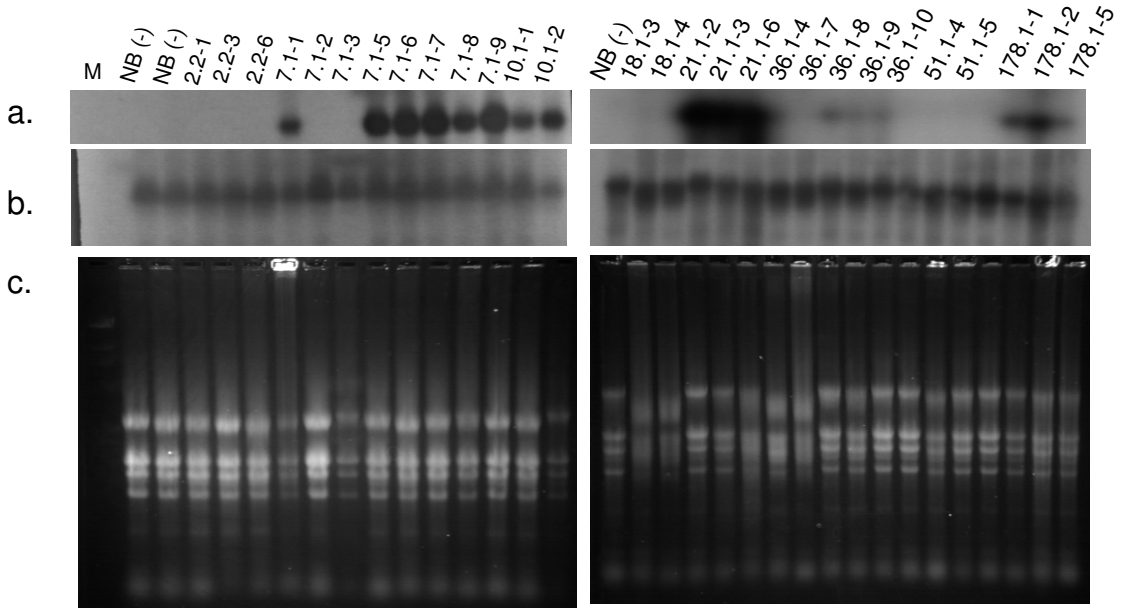

Figure 2.5. Developed film of Northern blot analysis for R1 lines. Northern blots were probed with a) 1.8 Kb EcoRI/EcoRI fragment of pSHX004 containing the caNPK1 coding domain, then stripped and probed with b) 18s probe. Original gel to show loading (c).

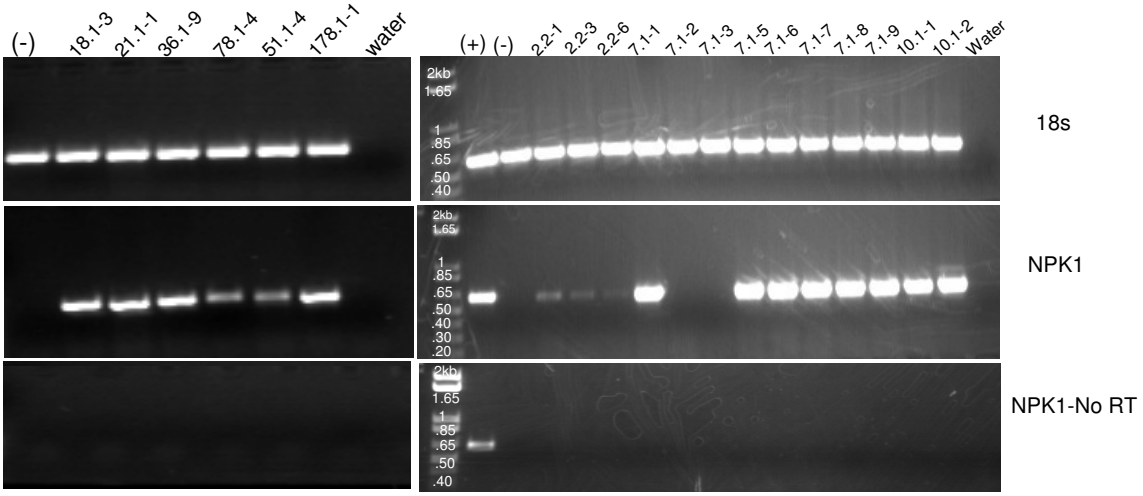

Figure 2.6. RT-PCR reaction of 18s and NPK1 fragment. A reaction designated "No RT uses NPK1-specific primers and is included as a control for genomic contamination. The positive control (+) is RNA spiked with plasmid pSHX004 DNA and is expected to amplify in all reactions. The negative control (-) is RNA from a Nipponbare (non-transgenic) plant.

Chapter 3: Physiological analysis of *caNPK1* transgenic rice under salt (sodium chloride) stress

Abstract

Genetic engineering offers a means for the improvement of agriculture in areas subject to saline soil conditions. Expression of the catalytic domain of NPK1 (caNPK1) increases salt tolerance in tobacco (Kovtun *et al.* 2000). Nipponbare rice transformed with tobacco *caNPK1* was grown in hydroponic culture. Four-week old transgenic and wild type plants were treated with moderate (150mM) or severe (300mM) salt stress. After four days of stress, physiological parameters related to salt-induced senescence, oxidative damage and ion homeostasis were measured. Visible signs of leaf damage, electrolyte leakage, malondialdehyde content, sodium ion content within leaves and sodium/potassium ratio did not significantly differ between transgenic and wild type rice. These results suggest that expression of caNPK1 may not impact metabolism or physiology of rice, or that kinase cascades, transcription factors, and functional proteins impacted by the activity of caNPK1 may relate to other stresses, namely pathogen response, drought or cold. Other aspects of salt stress response can be investigated further. In particular, the maintenance of yield components under prolonged stress or measurements of osmotic stress responses within the first minutes to hours of salt shock.

3.1 Introduction

3.1.1 The caNPK1 protein kinase and abiotic stress

Protein kinases, including the mitogen-activated protein kinases (MAPKs) are widely associated with response to biotic and abiotic stress (Ichimura *et al.* 2002; Nakagami *et al.* 2005; Pitzschke and Hirt 2006). *Nicotiana* Protein Kinase 1 (NPK1) is a mitogen-activated protein kinase kinase kinase (MAP3K) first described in connection with cell division (Nakashima *et al*. 1998; Nishihama *et al.* 2002; Soyano *et al.* 2003). Experiments using virus-induced silencing of NPK1 showed impaired disease resistance (Jin *et al*. 2002), suggesting a role in stress response. The constitutively active form of NPK1 (caNPK1) used in this study is the kinase domain of NPK1 under a constitutive auxin-insensitive promoter. The caNPK1 construct used to transform Nipponbare rice callus contains the same cassette used by Kovtun *et al*. (2000) to transform tobacco plants and Shou *et al.* (2004a,b) to transform maize. Studies expressing caNPK1 in tobacco indicated an increased tolerance to heat, cold, and salt in the transgenic plants (Kovtun *et al*. 2000). Expression of the same construct in maize resulted in plants more tolerant to cold and drought (Shou *et al*. 2004a,b). Additionally, in the absence of stress, caNPK1-expressing maize shows upregulation of stress-related genes particularly the antioxidant *glutathione-S-transferase* (*GST*), heat shock protein 17.8 (*HSP17.8*) and *pathogenesis-related protein 1* (*PR1*). Transcript levels of these genes increase in transgenic and non-transgenic maize following short-term cold exposure. (Shou *et al*. 2004a)

The stimulation of antioxidant and heat shock gene transcript accumulation in unstressed transgenic maize suggests the potential of caNPK1 to activate stress-response

pathways in the absence of stress. Salt tolerance studies using *caNPK1*-expressing maize were inconclusive because the plants, in soil, could not be dosed with salt as precisely as in a hydroponic system. Additionally, the hybrid nature of the Hi II transgenic maize results in the segregation of parental traits in each generation. This makes the distinction of tolerance and physical characteristics such as height and fertility difficult to attribute to the influence of caNPK1, rather than to intrinsic variability. Nipponbare rice, an inbred grass, is a model organism for the testing of salt stress and is more amenable to hydroponic culture. Therefore, Nipponbare rice carrying the caNPK1 cassette was used to assess the effect of caNPK1 gene expression upon salt tolerance. The studies described herein make use of a single insertion event with a high level of caNPK1 activity.

3.1.2 Salt stress physiology

Salt stress affects plants in two distinct phases. Phase 1 salt damage occurs within minutes or hours and may extend into days. Phase 1 shares many features also present during drought conditions (Hasegawa et al. 2000; Munns 2002). Damage that occurs to cells during phase 2 results from accumulation of sodium and chloride ions within the cells, damage which begins to occur in the oldest leaf after a period of days (Munns 2002, 2005). Cytoplasmic levels of Na^+ and Cl^- are kept low, often through sequestering the ions in the vacuole. High levels of Na^+ can inhibit K^+ uptake into cells (Hasegawa et al. 2000), therefore, the retention of discrimination between K^+ and Na^+ at high salt concentrations is often used in screening germplasm for salt tolerance (Munns and James 2003; Munns 2005). Sustained osmotic stress, coupled with the replacement of sodium

for potassium and toxic levels of sodium within the cell, leads to damage of the photosynthetic machinery (Fricke *et al.* 2006).

Engineering strategies for salt tolerance in rice have focused on maintaining favorable K^+/Na^+ status in the cytoplasm or the prevention of cellular damage through the production of osmolytes. Transgenic means of conferring ion homeostasis under salt stress include inducing increased sequestration of Na^+ in the vacuole by overexpression of genes encoding Na^+/H^+ antiporters, for example, *OsNHX1* (Bajaj and Mohanty 2005). Bacterial genes have been introduced into rice to encourage accumulation of the osmolytes proline and glycine betaine (Mohanty *et al.* 2002; Jang *et al.* 2003).

The influence of a constitutively active exogenous MAP3K upon osmolyte biosynthesis or cellular Na^+ concentration in whole plants has not yet been examined. Yeast and mammalian MAPK pathways have been attributed to upstream regulation of osmolyte biosynthesis (Xiong *et al.* 2002). *AtSOS1*, encoding an Arabidopsis Na^+/H^+ antiporter is regulated by a kinase, SOS2, and its accompanying calcineurin B-like calcium binding protein, SOS3 (Qiu *et al.* 2002). The ability of exogenous MAP3Ks to activate multiple MAP2Ks in host systems like yeast provides the opportunity for the stimulation of multiple pathways and potentially the creation of cascades not utilized in wild type plants (Kovtun *et al.* 1998; Covic *et al.* 1999).

3.1.3 Studies of salt stress

Measurements of several parameters and are put into place when screening cultivars for tolerance. Measures found to have the highest correlation with salt tolerance include electrolyte leakage and malondialdehyde (MDA) content. The most sensitive

cultivars show the highest percentages of electrolyte leakage and highest levels of MDA content during the first two weeks of exposure to salt stress (Lutts *et al.* 1996). Tolerant lines were found to have lower concentrations of Na^+ in leaf tissue and maintained a more favorable K^+/Na^+ ratio under salt stress, as seen in screening for salt tolerance among indica lines (Walia *et al.* 2005). Photsystem II efficiency during salt stress is frequently used as a physiological measure of salt tolerance, typically assessed via chlorophyll fluorescence measurements. Osmolytes may offer protection of the photosynthetic apparatus during stress conditions; studies of transgenic plants carrying bacterial genes for proline or glycine betaine biosynthesis pathways showed increased protection of photosystem II under salt stress Sakamoto *et al.* 1998; Garg *et al.* 2002; Mohanty *et al.* 2002). Transgenic maize expressing caNPK1 was capable of maintaining apparent photosynthetic rate under drought conditions (Shou *et al.* 2004b).

3.1.4 Activities undertaken described in this paper

Establishment of hydroponic system

A hydroponic system was prepared specifically for the application of salt stress to rice. A summary of the system and results from plants that have undergone a complete lifecycle within the system without stress are presented.

NaCl treatment

The incorporation of salt treatments into the hydroponic system was first tested exclusively on Nipponbare rice to determine whether the conditions established in our hydroponic culture were suitable for stress tests. The study deemed the pilot study observes the physiology of Nipponbare rice subjected to 0 mM, 150 mM or 300 mM to

determine how similar the results produced by our system are to those obtained in other publications.

Physiological measurements

Chlorophyll fluorescence measurements record the incident and reflected light and serve as an indicator of overall leaf health, with special reference to the activity of photosystem II. Electrolyte leakage occurs as a result of membrane permeability due to membrane damage. Malondialdehyde concentration quantification by the malondialdehyde assay (MDA assay) is a measure of the products of lipid peroxidation and an indicator of damage due to reactive oxygen. Spectrophotometric analysis of ion content reveals the accumulation of sodium ions within the leaf and, when compared to potassium ion levels in the leaf, may indicate late stage interruption of Na^+/K^+ transport.

3.2 Materials and Method

3.2.1 Hydroponic system

Seeds were pre-germinated by soaking in distilled water for 48 hours, then germinated on water-moistened filter paper in the dark at room temperature for 5 days. Following germination, the emerging seedlings were planted to Styrofoam flats, one plant per hole.

Styrofoam flats with circular holes were constructed to hold rice seedings in hydroponic culture. Plastic window mesh attached to the bottom of each Styrofoam flat prevented the seedling from falling through the hole. At planting, the mesh was covered with a layer of vermiculite and placed within the hydroponic tub to moisten the vermiculite. The seedling was gently transferred from the germination plate to the

vermiculite with forceps and then covered with more vermiculite until the seed itself was covered. An aquarium filter equipped with two rubber hoses supplied aeration for the roots. Fresh working solution was prepared weekly using 5 ml of stock solutions per 4 L distilled water and allowed to warm to 28°C within the growth chamber prior to transfer of flats to new solution. Hydroponic stock solutions: Solution I: 1.14 M NH_4NO_3, 798 mM $CaCl_2$; Solution II: 336 mM $NaHPO_4$, 410 mM K_2SO_4,; Solution III: 2.70 M $MgSO_4$; Micronutrient solution: 7.58 mM $MnCl_2 \cdot 4H_2O$, 598 nM $NH_4 6Mo_7O_{24} \cdot 4H_2O$, 15 mM H_3BO_3, 12.17 mM $ZnSO_4 \cdot 7H_2O$,12.42 mM $CuSO_4 \cdot 5H_2O$, 3.6mM $FeSO_4$)

Growth chamber (Conviron, USA) conditions for rice growth were 12 hours light/ 12 hours dark at average temperatures of 28°C during light and 25°C during dark. The light intensity was ~300 $\mu E/m^2 s$ at mid-canopy height.

3.2.2 Salt treatments

Germinated seedlings of Nipponbare or line 178.1 (the R_1 generation of caNPK1-transformed Nipponbare) were planted in nine flats. Three flats were randomly assigned to one of three treatments: 0 mM NaCl, 150 mM NaCl, 300 mM NaCl. Salt treatment was applied by preparing hydroponic solution as described in Table 1 with the addition of solid NaCl to the desired Molar concentration. The pH was then adjusted to 5.6 with KOH or HCl, as necessary. Long term stress treatment involved applying salt treatment 14 days after planting. Short term stress was applied to older plants (28 days after planting).

3.2.3 Chlorophyll Fluorescence reading

Five plants in each treatment were measured, using different plants on different days, using a PAM fluorometer (Waltz, Germany). Values for maximal variable fluorescence (Fv), basal (nonvariable) fluorescence (Fo) and maximum chlorophyll fluorescence (Fm) were recorded and the ratio of Fv/Fm calculated for each sample.

3.2.4 Electrolyte leakage measurement

Two leaves (second and third leaf from plants in four-leaf stage) were sampled. Each replicate included 7 mm diameter holes punched from two leaves from three plants (total of 6 leaf discs). The leaf discs were immersed in 15 ml double distilled water and shaken for 2 hours before initial conductivity was measured with a conductivity meter. Samples were then autoclaved and absolute conductivity measured.

3.2.5 MDA concentration measurement

Leaf blades were weighed and 0.25g of tissue per sample was immediately frozen in liquid nitrogen. The samples were ground in ceramic mortars with ceramic pestles (Coors, USA) with the constant addition of liquid nitrogen until a fine powder was obtained. To each sample of ground tissue was added 5 mL 0.1% TCA, the samples were centrifuged at 4,000 rpm for 30 min (Jouan). One ml of supernatant was then transferred to a fresh centrifuge tube and mixed with 4mL 20% TCA containing 0.5% TBA (Thiobarbituric acid) and then heated at 95^{o}C for 30 min. The samples were then immediately cooled on ice and centrifuged at 4,000 rpm for 10 min. For reading by spectrophotometer (KC4), 250 µl of supernatant were added to wells of flat-bottomed 96 well plate with 3 replicates per tube. Spectrophotometric readings of absorbance were

taken at 532nm and 600 with correction for a 1cm pathlength. The concentration of MDA within the sample is calculated using the following equation:

c (nmol MDA)= (A532-A600/155) x 1000

3.2.6 Ion content analysis

Three replicates of at least 3 g fresh weight were collected and dried for 5 days in a drying oven (38^{o}C). The samples were sent to the Plant and Soil Testing lab for sodium and potassium content determination using inductively coupled plasma-optical emission spectrophotometry. Results were converted from ppm to mM for comparison of means and Na+/K+ ratio .

3.2.7 Statistical analysis

Electrolyte leakage was calculated by expressing the ratio of initial conductivity to absolute conductivity as a percent. The percentages were converted to arcsin for data analysis. ANOVA was performed for each day separately and means compared using LS Means Differences (Student's t). Fv/Fm, Fo and Fm were analyzed with JMP software.to conduct analysis of variance with each day as a simple experiment. Means for chlorophyll fluorescence and ion content were each compared using LS Means Differences (Student's t).

3.3 Results and Discussion

3.3.1 Establishment of hydroponic system for rice stress treatment

The use of styrofoam 50 ml Falcon tube racks filled with vermiculite were determined to be the most convenient structure for hydroponic flats. The plants are

capable of tillering, flowering and setting seed in these containers (Table 3.1). Flag leaves appeared 56 days after planting. Changing of hydroponic solution was ceased after plants reached seed fill stage and instead distilled water added to prevent premature drying. When 25% of the panicles began to yellow, water addition was ceased. Drying and harvest was complete 4 months after planting. At late stages (seed fill, dry-down) lodging occurred in a few flats, but the plants could be righted by creating a frame around the aerial parts with strips of tape placed horizontally and vertically across the top of the hydroponic tub.

3.3.2 Pilot study using non-transgenic plants

Long term and short term salt stress was applied to plants 14 days after planting and continued for 32 days. Short term stress was applied to plants 28 days after planting and measurements were taken after 1, 2 and 4 days. Salt treatment was applied at levels of 0 mM, 150 mM, or 300 mM. At the end of 32 days, plants in 150 mM NaCl were showing damage to top leaves, although this was not reflected in the chlorophyll fluorescence measurements. Examples of leaf damage are pictured in Figure 3.1. Plants in 150 mM were discarded following final measures of electrolyte leakage and plants in 0 mM were kept to increase Nipponbare seed and to see the feasibility of bringing rice to flower in the hydroponic system. Plants exposed to 300 mM NaCl died quickly and measurements for long term stress could not be completed with the 300 mM treated plants.

Chlorophyll Fluorescence

Measurements of chlorophyll fluorescence stayed relatively steady over the duration of the stress with the exception of the plants in 300 mM NaCl at 4 days. Visible symptoms of leaf rolling and turning gray-green appeared as obvious signs of damage; the plants were dead by 7 days (Figure 3.1d). Efforts were made to adjust gain and intensity of the fluorometer to keep Fo between 300 and 400 mV. Despite attempts to control Fo, Fv/Fm measurements were consistently below 0.800 except for at 25 days of stress (Table 3.2). An Fv/Fm below 0.800 is typically an indicator of stress. At 25 days both control treated and 150 mM treated plants had Fv/Fm readings near 0.800.

Lutts et al. (Lutts *et al.* 1996) measured chlorophyll fluorescence components in salt tolerant and salt sensitive cultivars, with decreases in Fm and Fv/Fm in salt sensitive and moderately resistant lines after 18 days of treatment. In our experiments we used Nipponbare, a moderately resistant line, which from this data had decreases in Fv/Fm and Fm, but appeared to recover by 25 days. Chlorophyll fluorescence was not used in subsequent experiments because it did not quantify difference in moderate salt stress (150 mM) after 25 days.

Electrolyte leakage, MDA assays and ion concentration

During short term stress treatments, the damage to leaves was reflected in electrolyte leakage (Table 3.3). Electrolyte leakage increased more slowly in plants treated with 150 mM NaCl than in plants treated with 300 mM NaCl. Plants from the long term stress experiments did not show increased electrolyte leakage over time relative to the untreated controls. MDA concentration in leaves was measured on the fourth day of exposure to salt using plants from the short term salt stress. Levels of MDA increased

significantly above levels found in unstressed plants only in those treated with 300 mM NaCl (Figure 3.2). Following sampling for the MDA assays, the green tops of the plants from the short term stress experiments were harvested, dried and submitted for determination of ion concentration. Both sodium and potassium concentrations increase with salt concentration in the growth media (Figure 3.3), however, the rise of the Na+/K+ ratio to 0.14 in severely stressed rice compared to the ratio of 0.01 in unstressed rice may represent the loss of specificity of K+ transporters (Hasegawa *et al.* 2000).

The pilot study demonstrated the utility of electrolyte leakage and MDA assays in observing damage following short term stress applied to 1 month old plants. The visible increase in sodium concentration in leaf tissues indicated that plants grown under the set stress conditions were capable of reaching phase 2 salt damage.

3.3.3 Comparison of Salt Stress Responses between Transgenic and Wild Type

Following the pilot study, transgenic rice line 178.1 and wild type plants were grown together among six flats. Wild type and transgenic plants were randomized within each flat and two flats randomly assigned to salt treatments. Plants were grown in aerated hydroponic solution without salt until 28 days after planting. Salt treatments were applied by transferring two flats to new tubs of hydroponic solution containing 0 mM, 150 mM or 300 mM NaCl. After four days of treatment, damage to second and third leaves was assessed using electrolyte leakage assay as a measure of membrane damage and MDA content of fourth leaves as a measure of lipid peroxidation. Content of Na+ and K+ ions in the shoot were also measured.

Nipponbare is a moderately salt tolerant cultivar, capable of tolerating 100 mM NaCl (Hoai et al. 2003). A second, similar experiment used 100 mM NaCl as the moderate stress. No salt damage was seen on the lower leaves of 100 mM NaCl treated plants (wild type and transgenic) after 4 days, whereas in the first experiment this damage had occurred by 4 days at 150 mM (data not shown). Neither electrolyte leakage nor MDA content appeared to differ significantly between transgenic and non-transgenic plants (Figures 3.4 and 3.5).

Ion content measurements show no evidence of enhanced sodium exclusion or increased potassium uptake (Figure 3.6). Wild type plants did not increase potassium uptake under salt stress in this experiment. One key factor may be the use of a different formulation of iron in the hydroponic solution during this experiment than was used in the pilot experiment. Due to the pH of the iron formulation used in the pilot experiment, the media was brought to pH 5.6 with 5 ml 10N KOH into 24 L of solution, adding 2 mM K+ to the solution, raising the concentration of potassium in the solution from 1 mM to 3 mM. The hydroponic solution with the new iron formulation is brought to pH 5.6 with 5 ml HCl and the potassium concentration of the solution is 1 mM.

In the second experiment samples taken for electrolyte leakage came from the top and third leaves instead of the second and third leaves. Lower leaves acquire ion toxicity in the lower leaves first (Munns 2002). Sampling the young top leaf misses damage that could be occurring in the older leaves. Sampling for electrolyte leakage also removes a portion of the leaf end, where damage from salt is seen. The lack of these ends in the four out of six of the 300 mM-treated samples used in the MDA assay may have skewed the average MDA content and contributed to the large standard error.

3.4 Summary and recommendations

We have seen no conclusive improvement in salt tolerance in the transgenic relative to the non-transgenic plants either in appearance under salt stress or in quantifiable measures of damage. Primarily, salt stress over the course of hours has an osmotic and oxidative component in which MAPKs may be involved, but over the course of days results in ion toxicity which requires separate mechanism and kinases that may not be part of MAPK cascades. Specifically, the kinase salt overly-sensitive 2(SOS2) plays a role in the maintenance of Na^+/K^+ balance during salt stress through the activation of the Na^+/H^+ antiporter SOS1. SOS2 is a serine/threonine kinase, but may not be a downstream target of caNPK1 signaling (Qiu *et al.* 2002).

Production of antioxidants and compatible solutes are known to be downstream of MAPK cascades in yeast and mammals (Xiong *et al.* 2002), however, that connection is not definitively established in plants (Zhang *et al.* 2006). If osmolyte synthesis was increased by caNPK1 expression, a parameter other than PSII protection as measured by chlorophyll fluorescence would be recommended, as chlorophyll fluorescence measurements did not appear to accurately reflect the leaf health of wild type plants under salt stress.

Further investigation of caNPK1-expressing lines testing under sodium chloride stress could monitor responses to the osmotic portion of stress over the first few hours of salt shock with the inclusion of more lines with different levels of caNPK1 expression. A longer term study of the impact of salt on plant growth and reproduction such as tillering, height, time to flowering, and seed set in moderate salt stress (100mM or 120mM) vs. no additional sodium (Zeng and Shannon 2000). To separate the effects of sodium toxicity

from general osmotic stress and to test tolerance to different salts, several lines could be grown solutions containing Sorbitol, calcium chloride ($CaCl_2$), potassium chloride (KCl) or lithium chloride (LiCl) (Jin *et al.* 2005).

OsMEK1 shares >70% homology with NQK1, the substrate of NPK1 in tobacco. OsMEK1 has gene expression and protein activity inducible by moderate cold and drought but not salt or ABA (Wen *et al.* 2002). Due to the possibility of signaling through OsMEK1, we may expect the transgenic rice to show more pronounced differences in tolerance to drought than to salt and perhaps a stimulation in cold tolerance over moderate chilling temperatures. Further investigation of physiology focusing on more direct application of oxidative stress (e.g. paraquat treatment), application of ABA, and exposure to chilling conditions can dissect the portions of abiotic stress affected by expression of caNPK1.

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References

Bajaj, S. and Mohanty, A. (2005). Recent advances in rice biotechnology-towards genetically superior transgenic rice. Plant Biotechnology Journal **3**: 275-307.

Covic, L., Silva, N. F. and Lew, R. R. (1999). Functional characterization of ARAKIN (*ATMEKK1*): a possible mediator in an osmotic stress response pathway in higher plants. Biochemica et Biophysica Acta **1451**: 242-254.

Fricke, W., Akhiyarova, G., Wei, W., Alexandersson, E., Miller, A., Kjellbom, P. O., Richardson, A., Wojciechowski, T., Schreiber, L., Veselov, D., Kudoyarova, G. and Volkov, V. (2006). The short-term growth response to salt of the developing barley leaf. Journal of Experimental Botany **57**: 1079-1095.

Garg, A. K., Kim, J.-K., Owens, T. G., Ranwala, A. P., Choi, Y. D., Kochian, L. V. and Wu, R. J. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc. Natl. Acad. Sci. USA **99**: 15898-15903.

Hasegawa, P. M., Bressan, R. A., Zhu, J.-K. and Bohnert, H. J. (2000). Plant Cellular and Molecular Responses to High Salinity. Annual Review of Plant Physiology and Molecular Biology **51**: 463-499.

Hoai, N. T. T., Shim, I.-S., Kobayashi, K. and Usui, K. (2003). Accumulation of some nitrogen compounds in response to salt stress and their relationships with salt tolerance in rice (*Oryza sativa* L.) seedlings. Plant Growth Regulation **41**: 159-164.

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Kreis, M., Zhang, S., Hirt, H., Wilson, C., Heberle-Bors, E., Ellis, B. E., Morris, P. C., Innes, R. W., Ecker, J. R., Scheel, D., Klessig, D. F., Machida, Y., Mundy, J., Ohashi, Y. and Walker, J. C. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends in Plant Science **7**: 301-308.

Jang, I.-C., Oh, S.-J., Seo, J.-S., Choi, W.-B., Song, S. I., Kim, C. H., Kim, Y. S., Seo, H.-S., Choi, Y. D., Nahm, B. H. and Kim, J.-K. (2003). Expression of a Bifunctional Fusion of the Escherichia coli Genes for Trehalose-6-Phosphate Synthase and Trehalose-6-Phosphate Phosphatase in Transgenic Rice Plants Increases Trehalose Accumulation and Abiotic Stress Tolerance without Stunting Growth. Plant Physiol. **131**: 516-524.

Jin, H., Axtell, M. J., Dahlbeck, D., Ekwenna, O., Zhang, S., Staskawicz, B. and Baker, B. (2002). NPK1, an MEKK1-like Mitogen-Activated Protein Kinase Kinase Kinase, Regulates Innate Immunity and Development in Plants. Developmental Cell **3**: 291-297.

Kovtun, Y., Chiu, W.-L., Tena, G. and Sheen, J. (2000). From the Cover: Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. PNAS **97**: 2940-2945.

Kovtun, Y., Chiu, W.-L., Zeng, W. and Sheen, J. (1998). Supression of auxin signal transduction by a MAPK cascade in higher plants. Nature **395**: 716-720.

Lutts, S., Kinet, J. M. and Bouharmont, J. (1996). NaCl-induced Senescence in Leaves of Rice (*Oryza sativa* L.) Cultivars Differing in Salinity Resistance. Annals of Botany **78**: 389-398.

Mohanty, A., Kathuria, H., Sakamoto, A., Mohanty, P., Murata, N. and Tyagi, A. (2002). Transgenics of an elite indica rice variety Pusa Basmati 1 harbouring the *codA* gene are highly tolerant to salt stress. Theoretical and Applied Genetics **106**: 51-57.

Munns, R. (2002). Comparative physiology of salt and water stress. Plant, Cell and Environment 25: 239-250.

Munns, R. (2005). Genes and salt tolerance: bringing them together. New Phytologist **167**: 645-663.

Munns, R. and James, R. A. (2003). Screening methods for salinity tolerance: a case study with tetraploid wheat. Plant and Soil **253**: 201-218.

Nakagami, H., Pitzschke, A. and Hirt, H. (2005). Emerging MAP kinase pathways in plant stress signalling. Trends in Plant Science **10**: 339-346.

Nakashima, M., Hirano, K., Nakashima, S., Banno, H., Nishihama, R. and Machida, Y. (1998). The Expression Pattern of the Gene for NPK1 Protein Kinase Related to Mitogen-Activated Protein Kinase Kinase Kinase (MAPKKK) in a Tobacco Plant: Correlation with Cell Proliferation. Plant Cell Physiol. **39**: 690-700.

Nishihama, R., Soyano, T., Ishikawa, M., Araki, S., Tanaka, H., Asada, T., Irie, K., Ito, M., Terada, M., Banno, H., Yamazaki, Y. and Machida, Y. (2002). Expansion of the Cell Plate in Protein Cytokinesis Requires a Kinesin-like Protein/MAPKKK Complex. Cell **109**: 87-99.

Pitzschke, A. and Hirt, H. (2006). Mitogen-Activated Protein Kinases and Reactive Oxygen Species Signaling in Plants. Plant Physiol. **141**: 351-356.

Qiu, Q.-S., Guo, Y., Dietrich, M. A., Schumaker, K. S. and Zhu, J.-K. (2002). Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proc. Natl. Acad. Sci. USA **99**: 8436-8441.

Sakamoto, A., Murata, A. and Murata, N. (1998). Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. Plant Molecular Biology **38**: 1011-1019.

Shou, H., Bordallo, P., Fan, J.-B., Yeakley, J. M., Bibikova, M., Sheen, J. and Wang, K. (2004a). Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc. Natl. Acad. Sci. USA **101**: 3298-3303.

Shou, H., Bordallo, P. and Wang, K. (2004b). Expression of the *Nicotiana* protein kinase (NPK1) enhanced drought tolerance in transgenic maize. Journal of Experimental Botany **55**: 1013-1019.

Soyano, T., Nishihama, R., Morikiyo, K., Ishikawa, M. and Machida, Y. (2003). NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes & Development **17**: 1055-1067.

Walia, H., Wilson, C., Condamine, P., Liu, X., Ismail, A. M., Zeng, L., Wanamaker, S. I., Mandal, J., Xu, J., Cui, X. and Close, T. J. (2005). Comparative Transcriptional Profiling of Two Contrasting Rice Genotypes under Salinity Stress during the Vegetative Growth Stage. Plant Physiol. **139**: 822-835.

Wen, J.-Q., Oono, K. and Imai, R. (2002). Two Novel Mitogen-Activated Protein Signaling Components, OsMEK1 and OsMAP1, Are Involved in a Moderate Low-Temperature Signaling Pathway in Rice. Plant Physiol. **129**: 1880-1891.

Xiong, L., Schumaker, K. S. and Zhu, J.-K. (2002). Cell Signaling during Cold, Drought, and Salt Stress. The Plant Cell **Supplement 2002**: S165-S183.

Zhang, A., Jiang, M., Zhang, J., Tan, M. and Hu, X. (2006). Mitogen-Activated Protein Kinase Is Involved in Abscisic Acid-Induced Antioxidant Defense and Acts downstream of Reactive Oxygen Species Production in Leaves of Maize Plants. Plant Physiol. **141**:475-487.

Zeng, L. and Shannon, M. C. (2000). Salinity Effects on Seedling Growth and Yield Components of Rice. Crop Science **40**: 996-1003.

Plants grown in hydroponics
(planted 9/17/06, harvested 1/18/07)

	Tillers	Panicles	Seeds
Mean	20.8	15.61	132.8
SD	3.6	4.6	75.1
		n=5	

Table 3.1. Yield of plants grown in hydroponics. Control plants in the long term salt stress experiment were retired to set seed. At 56 days flag leaves began to appear. Plants were maintained in hydroponic solution that was changed weekly, then biweekly once flowering began. When plants had a large number of filled grains and the heads began to yellow the solution was no longer changed and water was added to prevent premature drying. Prior to harvest plants were left to dry completely. In total, 25 plants produced 60.6 grams of seed.

	Fv/Fm	(Mean of five replicates ± s.e)	
stress duration (d)	0mM NaCl	150mM NaCl	300mM NaCl
0	0.7368±0.0084	0.7326±0.0226	0.7296±0.0179
4	0.5750±0.0209[a]	0.622±0.0159[a]	0.1516±0.0850[b]
18	0.6846±0.0588	0.6386±0.0191	
25	0.8092±0.0250	0.7912±0.0079	

Fo	(Mean of five replicates ± s.e)	
0mM NaCl	150mM NaCl	300mM NaCl
0.2580±0.0168	0.2924±0.0214	0.3124±0.0223
0.4644±0.0331[a]	0.3840±0.0501[a]	0.1162±0.0186[b]
0.3260±0.0112[a]	0.2644±0.0138[b]	
0.2674±0.0101	0.2720±0.0082	

Fm	(Mean of five replicates ± s.e)	
0mM NaCl	150mM NaCl	300mM NaCl
0.9816±0.0611[a]	1.0964±0.0259[a,b]	1.1806±0.0791[b]
1.0928±0.0546[a]	1.0008±0.1016[a]	0.1532±0.0414[b]
1.0334±0.0343[a]	0.7458±0.0669[b]	
1.402±0.0578	1.3122±0.0788	

Table 3.2. Chlorophyll fluorescence measurements from pilot study. Fv/Fm, Fo and Fm measurements of wild type rice maintained in hydroponic solution under control (0mM NaCl) or salt treatments (150mM or 300mM NaCl). Letters indicate separation of means by LS Means Differences (α=0.05). Comparisons of means were conducted within individual days, not over time.

Electolyte leakage (%) (Mean of three replicates ± s.e) for long term stress and short term stress treatments

stress duration (d)	0mM NaCl	150mM NaCl	300mM NaCl	stress duration (d)	0mM NaCl	150mM NaCl	300mM NaCl
4	14.46±1.60[a]	18.15±1.31[a]	88.30±0.81[b]	1	5.24±0.23[a]	6.35±0.90[a]	22.91±11.79[b]
11	7.88±1.38	4.40±0.50		2	5.64±0.28[a]	7.71±0.46[a]	41.46±10.72[b]
18	5.62±0.51	9.58±2.18		4	23.15±0.81[a]	50.98±2.34[a,b]	77.1±4.04[b]
25	10.99±0.43	21.10±4.49					
32	5.40±0.99	11.61±3.32					

Results from one-way ANOVA at each day separately, long term stress and short term stress

	F Ratio	p-value		F Ratio	p-value
Day 4	652.71	<0.0001*	Day 1	466.326	<0.0001*
Day 11	6.6931	0.0609	Day 2	137.088	<0.0001*
Day 18	3.3029	0.148	Day 4	9.0378	0.0155*
Day 25	6.0959	0.069			
Day 32	3.9929	0.1164			

Table 3.3. Electrolyte leakage (%) of wild type plants during long term or short term salt stress.

Long term: With the exception of 300mM NaCl after 4 days, electrolyte leakage was not significant between treatments on any of the days.

Short term: Electrolyte leakage increased slower in 150mM NaCl-treated plants than those treated with 300mM NaCl. Measurements taken under 4 days may provide a quick assessment of stress tolerance when treatments are applied to transgenic and non-transgenic plants.

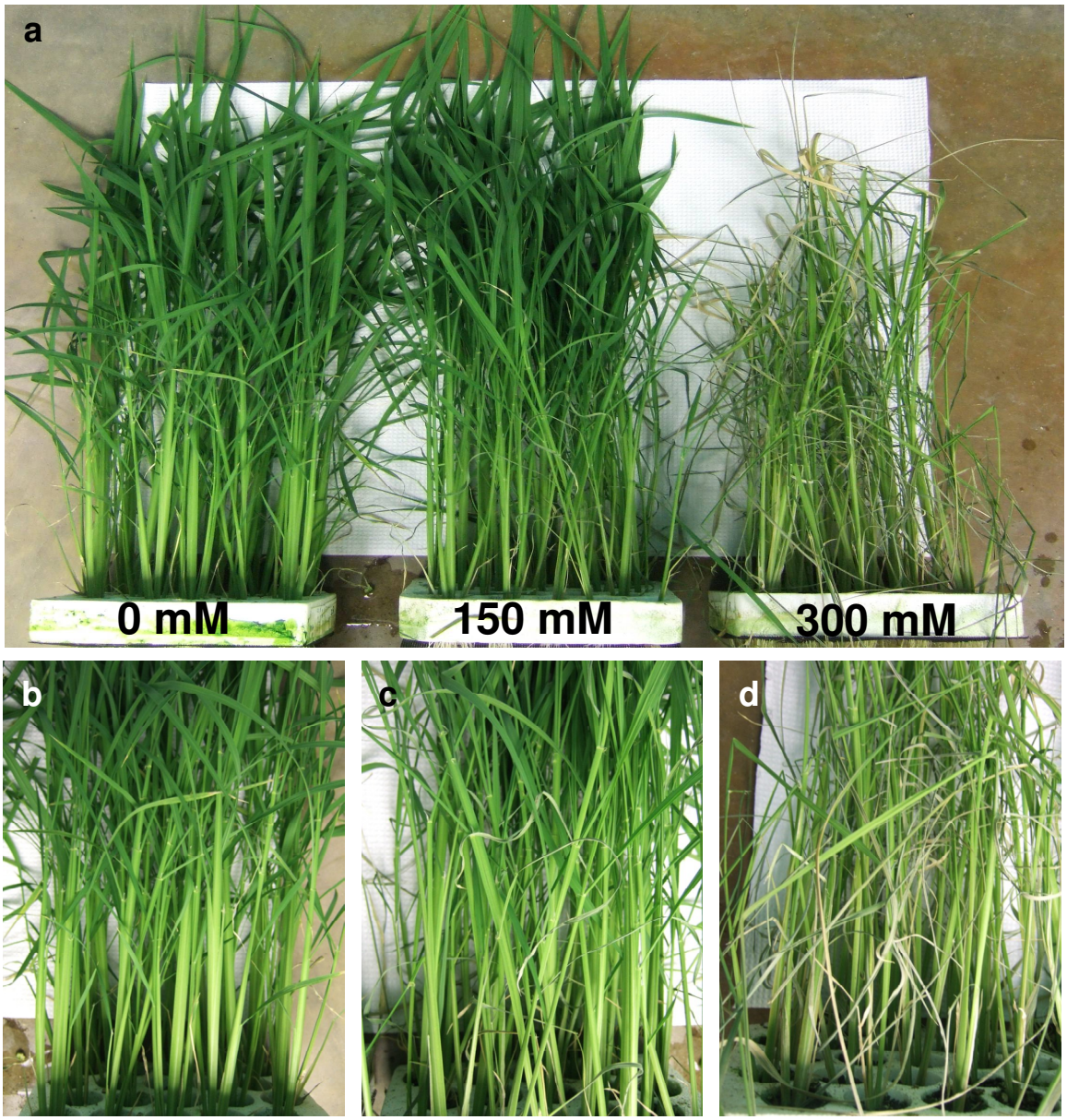


Figure 3.1. Visible leaf damage to Nipponbare plants after 4d NaCl treatment. Comparison of aerial portion (a). Close up of leaf damage (b-d) for 0 mM, 150 mM, 300 mM, respectively. Differences in apparent height between control and 300 mM treated plants was most likely due to the withering of leaves in the 300 mM treated flat. A small degree of damage was evident in the lower leaves of 150 mM treated plants (c). Withering and rolling of leaves in 300 mM NaCl began with the lower leaves, followed by wilting and graying and rolling of the tips of upper leaves. Plants that remained in 300 mM NaCl were completely dead after 7 days.

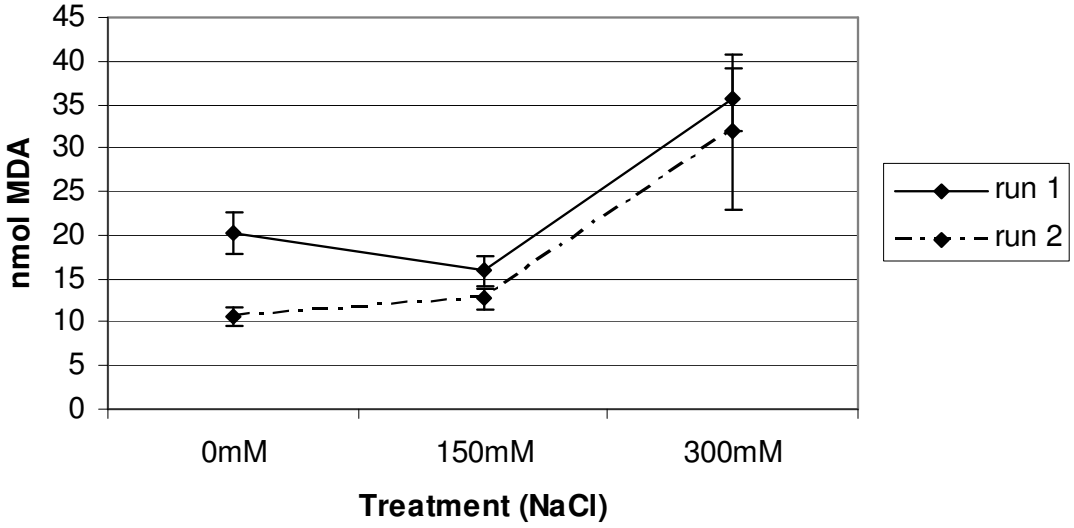
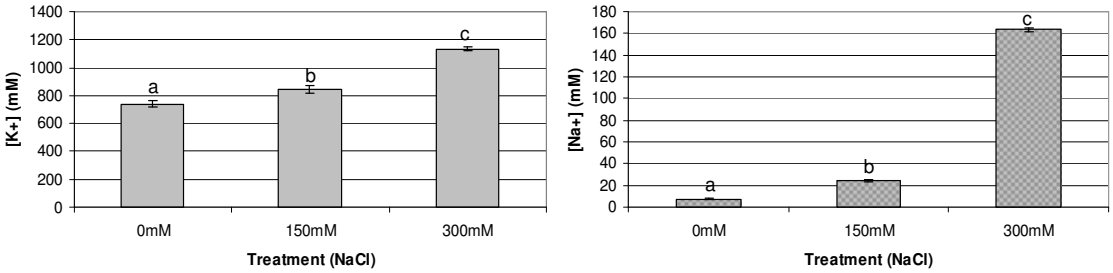


Figure 3.2. Initial MDA analyses of wild type exposed to salt treatments for 4 days. Three samples of each treatment were processed at two different times (run 1 and run 2). Samples were weighed at time of collection, but not after grinding, therefore the amount of tissue in each sample is assumed to be 0.25 g. Error bars are standard errors as calculated by Excel. The trends are the same, but differences in MDA concentration could come from differences in the weight of the tissue used in the assay.



	Difference in means	95% CI
K+		
150mM NaCl-0mM NaCl	103.33	(7.02, 199.64)
300mM NaCl-0mM NaCl	394.73	(298.42, 491.04)
300mM NaCl-150mM NaCl	291.40	(195.09, 387.71)
Na+		
150mM NaCl-0mM NaCl	16.96	(11.66, 22.27)
300mM NaCl-0mM NaCl	156.26	(150.95, 161.56)
300mM NaCl-150mM NaCl	139.29	(133.99, 144.60)

Figure 3.3. Ion concentration of wild type plants after 4 days exposure to salt. Means presented are based on three replicates. Oneway ANOVA analysis of $[K^+]$ by Treatment in JMP shows significant ($p<0.0001$) treatment effect. Tukey-Kramer HSD comparison of means shows significant differences in means at $\alpha=0.05$ (denoted by a,b,c). Similar results are seen with analysis of $[Na^+]$ by Treatment.

A

	Transgenic	Wild Type	Estimate	St. Error	95% CI
0mM*	19.956	17.005	+2.951	1.037	(0.072, 5.830)
150mM*	43.775	61.238	-17.462	7.899	(-39.395, 4.470)
300mM	58.042	69.365	-11.323	5.309	(-28.217, 5.572)

B

	Transgenic	Wild Type	Estimate	St. Error	95% CI
0mM*	23.212	19.033	+4.179	1.647	(0.150, 8.208)
100mM*	22.295	19.477	+2.817	1.288	(-0.334, 5.968)
300mM	60.379	63.839	-3.460	1.988	(-8.3240,1.403)

Figure 3.4. Means comparison of electrolyte leakage, (%) converted to arcsin, between transgenic and non-transgenic at each treatment level.

A) Differences between genotypes are not significant at the α=0.05 or α=0.10 level for means of 300 mM treatment.

*Means of transgenic and wild type plants differed significantly (one sided t-test, $p<0.05$) in control (0 mM) and 150 mM treatment. The higher value of electrolyte leakage in the control treatment could possibly be from an increased susceptibility to damage.

B) A second test of electrolyte leakage.

*Differences between genotypes are not significant at the α=0.05 or α=0.10 level for means of 300 mM treatment. The mean of the transgenic plants again showed a difference (one sided t-test, $p<0.05$) from wild type in the control (0 mM) treatment.

A

	Transgenic	Wild Type	Estimate	St. Error	95% CI
0mM*	3.71783	4.83922	-1.1214	0.419	(-2.285, 0.0418)
150mM	11.9426	14.5289	-2.586	3.102	(-11.199, 6.027)
300mM*	19.7156	24.1632	-4.448	2.035	(-10.098, 1.202)

B

	Transgenic	Wild Type	Estimate	St Error	95% CI
0mM	3.4437	5.0269	-1.5832	0.7578	(-3.6872, 0.5208)
100mM	3.9627	5.1540	-1.1914	1.3221	(-4.8622, 2.4795)
300mM	13.8017	11.8686	+1.933	8.670	(-22.140, 26.006)

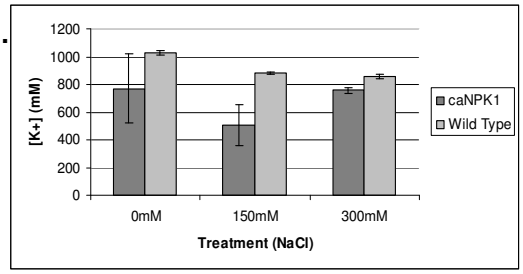
Figure 3.5. Comparison of means for MDA concentration between transgenic and nontransgenic rice after 4 days of salt treatment.

A) Comparison of means from initial comparison of salt stress responses.

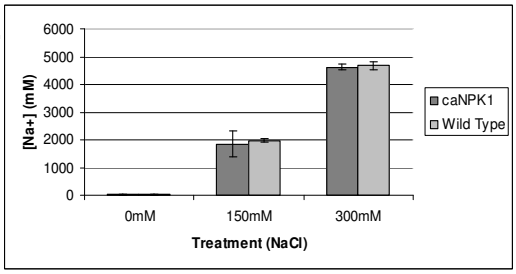
*Differences between genotypes are significant (one-sided $p<0.05$, $\alpha=0.10$) for 0 mM and 300 mM.

B) Comparison of means from second experiment. Differences between genotypes are not significant at the $\alpha=0.05$ or $\alpha=0.10$ level for any treatment.

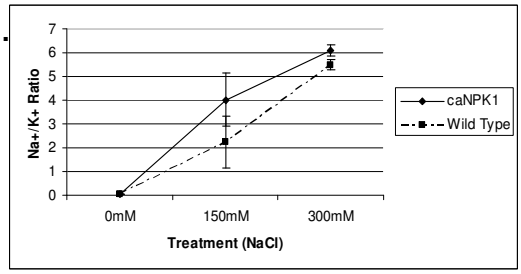
i.



ii.



iii.



iv.

[K+] (mM)	Means of three reps ± s.e		Difference in means		
	caNPK1	Wild Type	estimate	st err	95% CI
0mM	770.21 ± 248.04	1028.18 ± 15.85	-257.97	248.55	(-948.05, 432.12)
150mM	509.65 ± 146.77	881.88 ± 6.25	-372.22	146.91	(-780.12, 35.67)
300mM	757.24 ± 19.10	853.75 ± 16.63	-96.51**	25.33	(-166.82, -26.19)

**difference significant in two-tailed t-test (a=0.05)

[Na+] (mM)	Means of three reps ± s.e		Difference in means		
	caNPK1	Wild Type	estimate	st err	95% CI
0mM	23.24 ± 7.95	31.14 ± 12.03	-7.90	14.42	(-47.93, 32.13)
150mM	1849.18 ± 457.38	1971.89 ± 73.27	-122.70	463.20	(-1408.80, 1163.40)
300mM	4613.65 ± 101.49	4680.34 ± 130.30	-66.70	165.16	(-525.26, 391.87)

Na+/K+ ratio	Means of three reps ± s.e		Difference in means		
	caNPK1	Wild Type	estimate	st err	95% CI
0mM	0.0317 ± 0.00491	0.0303 ± 0.00491	+0.00136	0.01273	(-0.03398, 0.03671)
150mM	4.01 ± 1.11	2.24 ± 1.11	+1.7751	1.1128	(-1.3146, 4.8648)
300mM	6.10 ± 0.22	5.49 ± 0.22	+0.6126	0.3184	(-0.2714, 1.4965)

Figure 3.6. Ion content analysis of caNPK1 transgenic and wild type plants.
i) Potassium content did not appear to increase in plants exposed to salt. ii) Sodium ion accumulation rate and amount does not differ significantly between transgenic and wild type plants. iii) Changes in sodium/potassium ratio are similar between transgenic and wild type plants. iv) Means and comparisons of means for each ion and sodium/potassium ratio.

Chapter 4:

Transcriptomic analysis of genes induced by expression of constitutively active catalytic domain of *Nicotiana* Protein Kinase 1 gene in rice

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A manuscript prepared for submission to Theoretical and Applied Genetics.

Abstract

Stimulation of multiple stress-responsive genes through the expression of a single gene is a powerful option for engineering crops for enhanced stress tolerance. Signaling components such as mitogen-activated protein kinases (MAPKs) regulate stress-induced pathways. Expression of a constitutively active form of the tobacco MAPK kinase kinase *Nicotaina* Protein Kinase 1 (caNPK1) in rice alters gene transcript abundance in the absence of stress as assessed by microarray analysis. Genes potentially related to flowering and development are downregulated. Biotin biosynthesis and methionine recycling pathway genes appear to be upregulated in the transgenic plants. Thirteen transcripts with functions related to biotic and abiotic stress also show higher expression in *caNPK1*-expressing plants compared to wild type. Quantitative real-time PCR analysis of several known rice MAPKs indicates no significant difference in expression levels between *caNPK1* transgenic rice and wild type.

4.1 Introduction

The intersection of increasing population with exhaustion of arable land sets the theme for twenty-first century agriculture: economic and ecological sustainability, increased yields with minimal input (Pretty 2007). Genetic engineering has a place within the tools of sustainable agriculture. Current commercial applications of biotechnology allow for reduced, strategic use of inputs such as pesticide and herbicide (Eizaguirre et al. 2006). Breeding has increased the yield potential since the early twentieth century as displayed by record yields, however, environmental conditions still play the greatest factor in limiting realized crop yield (Boyer 1982), therefore, much effort is being aimed at engineering for protection against crop loss due to abiotic stress (Seki et al. 2003; Zhang et al. 2004; Bajaj and Mohanty 2005; Munns 2005; Vinocur and Altman 2005; Umezawa et al. 2006).

Transgenic and antisense experiments aimed at enhancing abiotic stress tolerance have increased survival and growth under specific stresses. Numerous reviews concerning the use of transgenic methods to enhance stress tolerance have been published (Seki *et al.* 2003; Zhang *et al.* 2004; Bajaj and Mohanty 2005; Munns 2005; Vinocur and Altman 2005; Umezawa *et al.* 2006). Within the laboratory, transgenic work provides insight into responses to environmental stress at the molecular level and provides targets and strategies for engineering tolerance, surmounting the barriers to maximal yield.

Protein kinases, including the mitogen-activated protein kinases (MAPKs) are widely associated with response to biotic and abiotic stress (Asai *et al.* 2002; Jonak *et al.* 2004; Boudsocq and Lauriere 2005; Nakagami *et al.* 2005). Members of the MAPK

family link the perception of the stress and gene transcription activated by transcription factors.

Each individual class of MAP kinase possesses different phosphorylation sites and characteristics. Phosphorylation of both the threonine and tyrosine residues in the activation loop of the MAPK by the MAPKK is necessary for activity. The activation loop of MAPKKs contains serine and/or threonine residues as phosphate acceptors from an active MAP3K (Ichimura et al. 2002). Plant MAP3Ks are more diverse in their activation mechanism, but retain the definitive function of phosphorylating substrate MAPKKs (Jouannic et al. 1999).

MAPK cascades can branch, with individual MAP3Ks activating multiple MAP2Ks, individual MAP2Ks activating multiple MAPKs, increasing the net downstream transcriptional and functional protein activation. Our laboratory has demonstrated the benefits of this strategy in maize (Shou *et al.* 2004a; 2004b) where constitutive expression of Nicotiana Protein Kinase 1, a MAP3K, enhanced freezing and drought tolerance in the transgenic maize plants.

The Mitogen-activated Protein Kinase Kinase Kinase (MAP3K) NPK1 (Banno *et al.* 1993) was first described in connection with cell division (Nakashima *et al.* 1998; Nishihama *et al.* 2002; Soyano *et al.* 2003). Virus-induced gene silencing of NPK1 resulted in abnormal cellular phenotypes in tobacco, but also impaired disease resistance (Jin *et al.* 2002) indicating a potential role in stress response. Kovtun et al (Kovtun *et al.* 1998) found expression of NPK1 under a constitutive auxin-insensitive promoter can activate MAPK proteins and also prevent activation of auxin-sensitive promoters in maize protoplasts. Expression of constitutively active NPK1 (*caNPK1*) in tobacco

increases tolerance to heat, cold, and salt (Kovtun et al. 2000). Maize expressing *caNPK1* are more tolerant to cold and drought and in the absence of stress and show upregulation of stress-related genes in an effect similar to short-term cold exposure (Shou *et al.* 2004a; Shou *et al.* 2004b).

Constitutive expression of the Arabidopsis NPK1 homolog, ANP1 (Krysan *et al.* 2002), in maize protoplasts led to the activation of H_2O_2-inducible promoters and expression of *caNPK1* enhanced tolerance of tobacco to cold, heat and salt (Kovtun *et al.* 2000). The stimulation of transcription seen in *caNPK1*-expressing plants may be related to crosstalk or the behavior of caNPK1 as a H_2O_2 signal mimic.

H_2O_2 and other ROS produced during stress serve as both a secondary messenger and as a component of stress-related damage (Xiong *et al.* 2002; del Rio *et al.* 2006; Gapper and Dolan 2006; Kwak *et al.* 2006). ROS are produced as a result of normal metabolism and, when highly regulated, mediate programmed cell death (PCD) and pathogen defense (Mittler 2002; Halliwell 2006). Conditions found to induce ROS production include drought, dessication, salt, chilling, heat shock, heavy metals, ultraviolet radiation, and ozone (Mittler 2002; Pitzschke and Hirt 2006).

Several mitogen-activated protein kinases (MAPKs) studied in Arabidopsis, tobacco and maize appear to act downstream of ROS signals (Pitzschke and Hirt 2006), however, MAPK activity preceding ROS generation has been described in tobacco (Sasabe et al. 2000; Yoshioka et al. 2003), Arabidopsis (Ren et al. 2002), and potentially as part of a positive feedback loop in maize (Zhang et al. 2006) where activity of a 46-kD MAPK is stimulated by H_2O_2 and also contributes to H_2O_2 production as well as antioxidant protein activity.

Expression of MAPK signaling components impacts gene expression. Expression of a constitutively active form of the Arabidopsis MAP2K, MKK2, in Arabidopsis increased expression of numerous transcription factors, defense genes and downregulated auxin-related genes (Teige *et al.* 2004). In maize expressing *caNPK1*, levels of *glutathione-S-transferase* (*GST*) gene expression were found to be nearly twofold higher in non-stressed transgenic plants than in their non-stressed null segregant siblings. A more pronounced increase in expression of *heat shock protein 17.8* (*HSP17.8*) and Pathogenesis-related (PR) protein gene *PR1* was seen in non-stressed transgenic plants (Shou et al. 2004b)

Based upon the research conducted in maize by Shou et al. (2004a,b), our hypothesis is that expression of *caNPK1* results in constitutive signaling through stress-related kinase cascades in the absence of stress. We desire to monitor differences in gene expression in multiple pathways between *caNPK1*-expressing and wild type plants using the Affymetrix Rice Genome Array. To this end, we have generated *caNPK1*-expressing Nipponbare rice and selected one transgenic line carrying a single insertion of *caNPK1* at a high level of transgene expression to use in microarray and quantitative Real-Time PCR studies. The study described herein represents the first survey, to our knowledge, of global transcriptional changes due to expression of an exogenous MAP3K in a crop species.

4.2 Materials and Methods

4.2.1 Plant material

Transgenic rice carrying the catalytic domain of NPK1 (caNPK1) under and auxin-insensitive promoter was generated by the Iowa State University Plant Transformation Facility. Nipponbare rice callus was transformed using Agrobacterium-mediated method with the construct pSHX004 (Shou et al., 2004a; Figure 4.1a). Of twenty-five independent transgenic lines, the line designated 178.1 was found to contain a single insertion of *caNPK1* and demonstrated a high level of *caNPK1* RNA expression as determined through Southern blot, Northern blot and RT-PCR analysis. Transgenic rice line R1S1 carrying a selectable marker *bar* gene and screenable marker uidA gene (pTF102, Frame *et al*., 2002) was included as control in analysis.

4.2.2 Plant growth conditions

Seeds were imbibed in distilled water for 48 hours and then germinated on filter paper for 5 days in the dark at 25^{o}C prior to planting. Germinated seedlings were planted to hydroponic flats. Within the growth chamber (Conviron, USA) 12 hour dark/12 hour light corresponded with average temperatures of 25^{o}C during the dark period and 28^{o}C during the light period. Light intensity measured ~300 μE/m^{2}s at mid-canopy height. Hydroponic solution, a dilution of 30 ml of each stock solution in 24 L distilled water (Stock Solution I: 1.14 M NH_4NO_3, 798 mM $CaCl_2$, Stock Solution II: 336 mM $NaHPO_4$, 410 mM K_2SO_4, Stock Solution III: 2.70 M $MgSO_4$, Micronutrient Stock Solution: 7.58 mM $MnCl_2{\cdot}4H_2O$, 598 nM $NH_46Mo_7O_{24}{\cdot}4H_2O$, 15 mM H_3BO_3, 12.17 mM $ZnSO_4{\cdot}7H_2O$, 12.42 mM $CuSO_4{\cdot}5H_2O$, 3.6 mM $FeSO_4$) was prepared fresh weekly and moderate

aeration was provided to the solution throughout the growing period. The top fully expanded leaf was collected for RNA isolation at 28 days after planting during tillering but before flag leaf emergence (Figure 4.2). Leaf samples for DNA isolation and PCR analysis were collected following collection of leaves for RNA isolation.

4.2.3 Experimental design for transcriptome analysis

R2 seedlings of line 178.1, R1 seedlings of the control transgenic line R1S1 and Nipponbare wild type were planted by random assignment among six hydroponic flats. To minimize impact of wounding on gene expression, tissue collection and RNA extraction were carried out as described below, prior to collection of leaf tissue for DNA extraction and PCR analysis. The R_2 generation of 178.1 and R_1 generation of R1S1 were still segregating. Transgenic segregants were determined through PCR. Equal amounts of RNA from four trangenic segregants of line 178.1 from a single flat were pooled. This process was repeated for each flat for a total of six *caNPK1* trangenic samples. Likewise, samples were prepared for the Nipponbare wild type for a total of six Nipponbare samples. Each sample was hybridized to a single Affymetrix Rice Genome Array (Figure 4.3). RNA from transgenic segregants of line R1S1 were reserved for quantitative reverse transcriptase (qRT)-PCR analysis to serve as controls for transformation with the bar gene.

4.2.4 PCR to identify transgenic segregants

DNA from leaves was isolated from a 1 inch long section of leaf tip from the second leaf. Leaf samples were kept on ice after collection and homogenized with 600 µl DNA extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5%

SDS), ground well with autoclaved kimble pestles. The homogenized sample underwent phenol and chloroform extractions followed by isopropanol precipitation and ethanol wash. The dried pellet was resuspended in 50 µl TE 10/0.1 + 2 µl RNAse A. Optimum conditions for the detection of the *bar* and NPK1 genes differ and were determined separately. Primer sequences used for this analysis: NPK1int-f: 5'-TAA CAA ATG GAT GCT GAA GC-3'; NPK1int-r: 5'-CCA TCC CAA CAT AGT GAG AT-3'; Bar11: 5'-CAG CTG CCA GAA ACC CAC GT -3'; Bar12: 5'- CTG CAC CAT CGT CAA CCA CT-3'.

4.2.5 RNA isolation

Total RNA was isolated from whole leaves representing the top fully expanded leaves of Nipponbare, Bar gene PCR-positive plants of line R1S1 and *caNPK1* PCR-positive plants of line 178.1. Snap frozen leaf tissue at an approximate weight of 100 mg was ground in ceramic mortar and pestles (Coors, USA) with constant application of liquid nitrogen. RNA extraction from the ground leaf tissue was done according to the Trizol method. Quantity of the RNA was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Quality of the RNA was checked with non-denaturing agarose gel electrophoresis.

4.2.6 Transcriptome analysis

Transcriptome analysis was performed using the GeneChip Rice Genome Array from Affymetrix (Santa Clara, CA). This array contains oligonucleotide probesets to query approximately 50,000 transcripts, of which 48,564 transcripts represent the japonica cultivar and 1,260 transcripts from the indica cultivar. Processing of the RNA

samples, hybridization, washing and preliminary analysis was performed by the GeneChip facility at Iowa State University.

Normalization and conversion of signal data to expression data was done using R software and the BioConductor application (www.r-project.org). Signal data from the genechips was converted to expression data using both the Robust Multichip Average (RMA) analysis method (Irizarry *et al.* 2003) and the Microarray Suite 5.0 (MAS5.0) algorithm, separately. Gene expression data was analyzed for treatment (genotype) effect with mixed proc in SAS (SAS Institute, USA) by fitting a model with random replication effects and fixed genotype effects. The p-values resulting from t-tests of genotype effect were converted to q-values (Storey and Tibshirani 2003). Genes showing at least a 1.5-fold expression difference between wild type and *caNPK1* transgenic and within the false discovery rate (FDR) threshold of 20% (q-value $\leq$ 0.20) were included in the final data set and researched for additional annotation and functional classification in NCBI (www.ncbi.nlm.nih.gov) and the Knowledge-based Oryza Molecular biological Encyclopedia (KOME) rice cDNA clone accession database (http://cdna01.dna.affrc.go.jp/cDNA/ANNOTATE/annotate_ accession_browse.html).

4.2.8 qRT-PCR

Relative quantitation of real-time PCR products was carried out using SYBRGreen chemistry (Invitrogen) on an ABI Prism 7900 (Applied Biosystems, USA). Total RNA from *caNPK1* transgenics, *bar*/*uidA* transgenics and Nipponbare wild type was converted to cDNA using Superscript III enzyme (Invitrogen) according to the manufacutrer's instructions. Primer sequences for 17 rice MAPKs (MPK1-17) identified

by Reyna and Yang (2006) and primers against 9 selected genes found to be significantly upregulated or downregulated in *caNPK1* transgenic plants were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, Iowa). Expression levels were determined by relative quantification (ΔΔCt method). Gene expression in at least two cDNA samples from *caNPK1* transgenic, wild type and R1S1 (*bar/uidA* control) plants were measured. Phospholipase D (*OsPLD*) served as the normalizer (Protocol, E.C.). A single wild type sample served as calibrator. Primers used are listed in Table 4.1.

4.3 Results and discussion

RNA samples were applied to twelve Affymetrix Rice Genome Arrays. Six arrays were hybridized with RNA samples from caNPK1 transgenic plants and the remaining six with RNA from wild type plants. Transcripts showing statistically significant differences in means and fold change ratios greater than 1.5 were functionally categorized by BlastP search of the transcript public ID.

Genes of interest were highlighted from the microarray data first by placing a threshold on statistical significance and then on potential biological significance. Using the gene expression data generated by RMA analysis, a primary gene list was made which contained genes with statistically significant differences in expression between *caNPK1* transgenics and Nipponbare wild type (p-value <0.001, q-value <0.20). The list was then limited to genes with differences in expression greater than 1.5-fold in data first normalized by RMA or MAS5.0. Tables 4.2 and 4.3 show genes downregulated and

upregulated in *caNPK1* transgenics, respectively. The fold change ratio value obtained from expression data produced through both RMA and MAS5.0 is shown.

4.3.1 Stress related genes are upregulated in transgenic plants in the absence of stress

Genes upregulated in *caNPK1* transgenics can be classed into thirteen categories (Figure 4.4a). The largest percentage (54%) of upregulated genes falls into the class of unknown or hypothetical proteins, followed by 13 transcripts which form a diverse group of stress-related proteins. In addition, of four kinases upregulated in *caNPK1* transgenics, three are putative. In general, genes downregulated in *caNPK1* transgenics are grouped into eight categories. Fewer genes appear to be downregulated (Figure 4.4b), 27, compared to 99 upregulated genes. The greatest percentage belongs to the unknown and hypothetical proteins, but four flowering and reproductive associated genes, including a MADS-box protein make up the highest percentage of downregulated genes with functional annotation. Kinases downregulated in *caNPK1* transgenic plants appear to be in the calcium-dependent protein kinase family (CDPK11, putative CDPK-related protein).

A large percentage of genes identified as differentially expressed between wild type and *caNPK1* transgenic fall into the class of hypothetical or unknown proteins. Of the array elements with annotations indicating functions, the following trends emerged:

Downregulation of floral and reproduction-associated genes-

Three of the 27 transcripts downregulated in caNPK1 transgenic plants may play roles in flowering or reproduction. These transcripts have high similarity to S-locus glycoproteins, a MADS transcription factor and the no-apical meristem transcription

factor. The S-locus glycoproteins are highly studied in *Brassica* relative to their role in self-incompatibility (Takayama and Isogai 2003). In maize, ZmPK1 was described as a Receptor-like kinase with an extracellular domain homologous to *Brassica* S-locus glycoprotein (Walker and Zhang 1990). No characterization of an S-locus glycoprotein-like protein or ZmPK1 homolog in rice have yet been published. The function of the putative S-locus glycoprotein family protein identified by the array is unknown.

The remaining two genes in the category of reproduction/floral development are matches to transcription factors of the MADS-box protein family and NAM (no apical meristem) transcription factors. NAM transcription factors are classified within the NAC family of transcription factors, which in Arabidopsis have members that are downstream of auxin signaling (Guo *et al.* 2005; He *et al.* 2005). In Arabidopsis, MADS-box proteins have also demonstrated auxin-responsive behavior (Zhu and Perry 2005).

MADS-box transcription factors regulate floral development. Comparison of MADS-box factor functions show conserved activities between Arabidopsis and rice (Kater *et al.* 2006). Research on the role of MADS-box proteins in flowering of *indica* rice identifies MADS57 as primarily found in mature leaves and not among the MADS-box genes expected to be involved in panicle development and seed set. Additionally, MADS57 was not among the MADS-box genes found to change expression by more than twofold following cold, dehydration, or salt stress (Arora *et al.* 2007). It remains unknown whether the role of MADS57 in vegetative tissue has an auxin-responsive component.

The No apical meristem (NAM) family of transcription factors were first characterized in petunia and show mRNA accumulation near the boundaries of meristems

and primordial (Souer *et al.* 1996). In monocots, NAM transcription factors identified in maize have roles in establishment of the shoot apical meristem. Other members of the family to which NAM transcription factors belong display mRNA expression localized to coleorhizae during embryogenesis (Zimmermann and Werr 2005). Neither AK066107, nor BAD53136.1 produced no substantial similarities to the ZmNAC or ZmNAM proteins identified by Zimmermann and Werr (2005).

Downregulation of calcium-dependent protein kinases-

Calcium-dependent protein kinases (CDPKs) represent the largest family of calcium signaling kinases in plants (Harper *et al.* 2004). In rice, numerous CDPKs are responsive to abiotic stress (Saijo *et al.* 2000; Ray *et al.* 2007; Wan *et al.* 2007) and hormone responsive (Abo-El-Saad and Wu 1995; Yang and Komatsu 2000; Abbasi *et al.* 2004). CDPK11, first cloned and characterized by Breviario *et al*. (1995), does not appear responsive to anoxic stress (Breviario *et al.* 1995), dessication, salt stress or cold stress and additionally is unchanged during the development of panicles and seed in indica variety IR64 (Ray *et al.* 2007). The precise function of OsCDPK11 remains unclear.

Upregulation of stress-associated genes-

Thirteen transcripts were grouped under the functional characterization of stress-associated. These transcripts bore amino acid sequence similarity to MAWD binding protein, lipase, cytochrome P450 family members, a putative salt tolerance protein, dirigent proteins, Spl7b, rhodanese-like domain containing proteins, a putative far-red impaired response protein, rhp1, and an NB-ARC domain.

A transcript similar to MAWD-binding protein of rice was found in the roots of barley exposed to phyotoxic levels of boron (Patterson *et al.* 2007). Cytochrome P450 family members are upregulated in Arabidopsis and barley in response to salt stress (Ma *et al.* 2006; Ueda *et al.* 2006), in rice in response to senescence and nutrient deficiency (Sperotto *et al.* 2007), and in response to oxidative stress in Arabidopsis (Desikan *et al.* 2001). Loss of function of the gene rhp16 in yeast (S. pombe) led to increased susceptibility to UV (Lombaerts *et al.* 1999). Spl7 is a heat shock transcription factor protein (Yamanouchi *et al.* 2002).

Dirigent proteins are involved in lignan production, but also may be defense-related, as they have been described in conifers in relation to roles in wounding response and insect defense (Ralph *et al.* 2006). The NB-ARC domain is a component of LRR-NBS R-proteins, proteins involved in plant innate immunity (Takken *et al.* 2006). Rhodanese domains are found among many eukaryotes and play diverse roles in cellular defense, metabolism and cell cycle regulation (Cipollone *et al.* 2007). In plants, rhodanese-like domain containing proteins have been isolated from cucurbit sap ad deemed stress- and defense-responsive (Walz *et al.* 2004). The rhodanese-like domain was identified within Arabidopsis thaliana SIR1. Loss of function mutants, sir1, were auxin hypersensitive, suggesting SIR1 is a negative regulator of auxin signaling (Zhao *et al.* 2007).

Upregulation of kinases-

Four proteins with putative kinase function appear to be upregulated in *caNPK1* transgenic plants. The sequence of the putative wall-associated protein kinase did not return sequence identities with previously described rice kinases through search using

BlastP. Os.19044.1.S1.at shared homology with CA764426, protein kinase-like domain containing protein also shared homology with R2R3 Myb protein, a member of stress-responsive transcription factors.

Upregulation of biotin and methionine metabolism-

The biotin synthesis and methionine recycling pathways may be interplaying in *caNPK1*-expressing rice. The first committed step of biotin biosynthesis, condensation of L-alanine and pimeloyl-CoA to form 8-amino-7-oxononanoate by 8-amino-7-oxononanoate synthase (EC 2.3.1.47) (Alexeev *et al.* 1998). The second step in biotin synthesis, catalyzed by adenosylmethionine 8-amino-7-oxononanoate aminotransferase (EC 2.6.1.62), releases S-adenosyl-4-methylthio-2-oxobutanoate as a byproduct.

Enzymes involved in methionine recycling are also apparently upregulated. Whether this is in response to recycling S-adenosyl-4-methylthio-2-oxybutanoate will still need to be investigated. S-adenosylmethionine synthetase 1 (EC 2.5.1.6) catalyzes the synthesis of S-adenosylmethionine, which may be used in the synthesis of biotin, but is also involved in the synthesis of polyamines. Polyamines play diverse roles in cellular functions including secondary metabolism, cell division, senescence and apoptosis (Yang *et al.* 2007). The polyamines spermine and spermidine which require S-adenosylmethionine for their biosynthesis are related to drought tolerance in rice and may play a role as antioxidants during protection of cells from various abiotic stresses (Groppa and Benavides 2007; Yang *et al.* 2007). S-adenosylmethionine can also be recycled through the methionine recycling pathway (Baur and Yang 1972), of which aci-reductone dioxygenase (EC 1.13.11.54) is a part.

4.3.2 Expression profile of rice MAPKs in transgenic vs. non-transgenic plants in the absence of stress

Several stress-responsive MAPKs in rice are transcriptionally activated by stress (Agrawal *et al.* 2003c). Expression levels of the majority of rice MAPK genes tested did not differ significantly, statistically or biologically between *caNPK1* transgenics and Nipponbare (Figure 7). The limited number of MAPKs with average expression either 2-fold greater or 2-fold less than the calibrator sample and significantly different (a=0.05) than the average of three Nipponbare samples suggests that the presence of *caNPK1* does not cause numerous, non-specific alterations of MAPK transcription.

Of the 17 putative MAPKs (OsMPK1-17) used in studies by Reyna and Yang (2006), several have been previously characterized as biotic or abiotic stress responsive. Fungal infection induced OsMPK5 and OsMPK12 and an additional seven previously uncharacterized putative MAPKs (OsMPK2, OsMPK4, OsMPK7, OsMPK8, OsMPK13, OsMPK15, OsMPK17). If caNPK1 mimics an oxidative stress signal in the absence of stress we would expect several of the MAPKs induced by blast fungus to have increased gene expression in *caNPK1*-expressing rice relative to wild type. No differences in level of gene expression had been observed for OsMPK2, OsMPK4, OsMPK7, OsMPK8 or OsMPK13 (Figure 7).

4.5. Summary and Conclusions

4.5.1 Microarray analysis

Kovtun *et al.* (2000) postulate that a constitutively active Arabidopsis homolog of NPK1 (caANP1) and possibly caNPK1 act as a mimic of the H_2O_2 signal, a component of

both biotic and abiotic stress and can suppress auxin signaling (Kovtun *et al.* 1998). Genes within the stress-repsonse category find function or expression linked to abiotic stress or cell damage and those related to wounding, herbivore and pathogen stress, however, no overall pattern in stress responsive proteins emerges. There also is neither upregulation of oxidative-stress induced genes within antioxidant defense, such as *gluthione S-transferase* (Shou *et al.* 2004a) nor downregulation of clearly auxin-responsive genes as might be expected (Kovtun *et al*. 1998, 2000; Teige *et al.* 2004). There were very few stress-responsive transcription factors, and none within the WRKY family, which are expected to be downstream of MAPK signaling cascades (Asai et al. 2002; Teige et al. 2004).

The role of biotin synthesis and methionine recycling in the metabolic condition of *caNPK1* plants may be illuminated by delving into the data using different analysis methods. A transcriptomic profile with such relevance to genetic engineering strategies as well as basic understanding of kinase cascades in rice such as the ectopic expression of a foreign kinase may warrant further analysis of this dataset with analysis methods that make use of gene ontology (GO) (Barry *et al.* 2005; Liu *et al.* 2007).

4.5.2 Confirmation of array results

Confirmation of gene expression changes with qRT-PCR remain to be completed. These genes and their primer sequences appear in Table 4.4. Gene expression will be represented as relative quantification against OsPLD as the endogenous control and calibrated to one of three Nipponbare wild type samples. Of primary interest are those with putative stress-tolerance roles (*putative dehydration-responsive protein*, *putative salt tolerance protein*, *putative dirigent protein*), potential kinase functions (*protien kinase-*

like domain containing/R2R3 Myb protein gene, *CDPK11*, *putative wall-associated kinase*). *Rhodanese-like domain containing protein is of interest* due to its potential connection to auxin regulation. *S-adenosylmethionine synthetase 1 serves* as a representative of the methionine/biotin biosythesis pathways. Potential *match to 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase* was selected for expression level confirmation due to its part in the shikimate pathway, a pathway that may be modulated by the presence of the *bar* gene.

4.5.3 Effect of caNPK1 expression upon OsMPK gene expresion

In tobacco, NPK1 phosphorylates NQK1 to regulate cell division (Soyano *et al.* 2003). NQK1 and OsMEK1 (Wen *et al.* 2002) share 78% amino acid identity as determined by amino acid sequence alignment (AlignX, Vector NTI Suite). Signaling in *caNPK1* plants could potentially be mediated by OsMEK1. One identified downstream substrate of OsMEK1 is OsMAP5 (also called OsMAP1) (Wen *et al.* 2002). It is conceivable that signaling mediated through OsMEK1 could be displayed in increased transcription of OsMAP5. OsMAP5, OsMPK11, and OsMPK15-17 transcript levels must be re-examined as the reaction conditions used for OsMAP1-4 and 6-14 were not ideal for amplification with OsMAP5 primers.

While it appears that *caNPK1* expression had no impact on the expression of the rice MAPKs analyzed by qRT-PCR, caNPK1 may be active in post-translational modification resulting in kinase signaling. Testing interactions of caNPK1 with rice MAP2Ks can shed light on the signaling dynamics of *caNPK1* expression in rice. Work utilizing yeast two-hybrid assays for protein-protein interactions are being undertaken to

assess interaction of caNPK1 with OsMEK1 and multiple putative rice MAP2Ks identified within the KOME database.

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References

Abbasi, F., Onodera, H., Toki, S., Tanaka, H. and Komatsu, S. (2004). *OsCDPK13*, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. Plant Molecular Biology **55**: 541-522.

Abo-El-Saad, M. and Wu, R. (1995). A Rice Membrane Calcium-Dependent Protein Kinase Is Induced by Gibberellin. Plant Physiol. **108**: 787-793.

Agrawal, G. K., Agrawal, S. K., Shibato, J., Iwahashi, H. and Rakwal, R. (2003a). Novel rice MAP kinases OsMSRMK3 and OsWJUMK1 involved in encountering diverse environmental stresses and developmental regulation. Biochemical and Biophysical Research Communications **300**: 775-783.

Agrawal, G. K., Iwahashi, H. and Rakwal, R. (2003b). Rice MAPKs. Biochemical and Biophysical Research Communications **302**: 171-180.

Agrawal, G. K., Rakwal, R. and Iwahashi, H. (2002). Isolation of novel rice (*Oryza sativa* L.) multiple stress responsive MAP kinase gene, *OsMSRMK2*, whose mRNA accumulates rapidly in response to environmental cues. Biochemical and Biophysical Research Communications **294**: 1009-1016.

Agrawal, G. K., Tamogami, S., Iwahashi, H., Agrawal, V. P. and Rakwal, R. (2003c). Transient regulation of jasmonic acid-inducible rice MAP kinase gene (*OsBWMK1*) by diverse biotic and abiotic stress. Plant Physiology and Biochemistry **41**: 355-361.

Alexeev, D., Alexeeva, M., Baxter, R. L., Campopiano, D. J., Webster, S. P. and Sawyer, L. (1998). The Crystal Structure of 8-Amino-7-oxononononanoate Synthase: A

Bacterial PLP-dependent, Acyl-CoA-condensing Enzyme. Journal of Molecular Biology **284**: 401-419.

Arora, R., Agarwal, P., Ray, S., Singh, A. K., Singh, V. P., Tyagi, A. K. and Kapoor, S. (2007). *MADS-box* gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics **8**.

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F. M. and Sheen, J. (2002). MAP Kinase signalling cascade in *Arabidopsis* innate immunity. Nature **415**: 977-983.

Bajaj, S. and Mohanty, A. (2005). Recent advances in rice biotechnology-towards genetically superior transgenic rice. Plant Biotechnology Journal **3**: 275-307.

Banno, H., Hirano, K., Nakamura, T., Irie, K., Nomoto, S., Matsumoto, K. and Machida, Y. (1993). *NPK1*, a Tobacco Gene That Encodes a Protein with a Domain Homologous to Yeast BCK1, STE11, and Byr2 Protein Kinases. Molecular and Cellular Biology **13**: 4745-4752.

Baur, A. H. and Yang, S. F. (1972). Methionine metabolism in apple tissue in relation to ethylene biosynthesis. Phytochemistry **11**: 3207-3214.

Boudsocq, M. and Lauriere, C. (2005). Osmotic Signaling in Plant. Multiple Pathways Mediated by Emerging Kinase Families. Plant Physiol. **138**: 1185-1194.

Boyer, J. S. (1982). Plant Productivity and Environment. Science **218**: 443-448.

Breviario, D., Morello, L. and Giani, S. (1995). Molecular cloning of two novel rice cDNA sequences encoding putative calcium-dependent protein kinases. Plant Molecular Biology **27**: 953-967.

Cheong, Y. H., Moon, B. C., Kim, J. K., Kim, C. Y., Kim, M. C., Kim, I. H., Park, C. Y., Kim, J. C., Park, B. O., Koo, S. C., Yoon, H. W., Chung, W. S., Lim, C. O., Lee, S. Y. and Cho, M. J. (2003). BWMK1, a Rice Mitogen-Activated Protein Kinase, Locates in the Nucleus and Mediates Pathogenesis-Related Gene Expression by Activation of a Transcription Factor. Plant Physiol. **132**: 1961-1972.

Cipollone, R., Ascenzi, P. and Visca, P. (2007). Common themes and variations in the rhodanese superfamily. IUBMB Life **59**: 51-59.

del Rio, L. A., Sandalio, L. M., Corpas, F. J., Palma, J. M. and Barroso, J. B. (2006). Reactive Oxygen Species and Reactive Nitrogen Species in Peroxisomes. Production, Scavenging, and Role in Cell Signaling. Plant Physiol. **141**: 330-335.

Desikan, R., Mackerness, S. A. H., Hancock, J. T. and Neill, S. J. (2001). Regulation of the *Arabidopsis* Transcriptome by Oxidative Stress. Plant Physiol. **127**: 159-172.

Eizaguirre, M., Albajes, R., Lopez, C., Eras, J., Lumbierres, B. and Pons, X. (2006). Six years after the commercial introduction of Bt maize in Spain: field evaluation, impact and future preospects. Transgenic Research **15**: 1-12.

Frame, B.R., Shou, H., Chikwamba, R., Zhang, Z., Xiang, C, Fonger, T., Pegg, S-E., Li, B., Nettleton, D., Pei, P., Wang, K. *Agrobacterium*-mediated transformation of maize embryos using a simple binary vector system. Plant Physiology, **129**:13-22 (2002).

Fu, S.-F., Chou, W.-C., Huang, D.-D. and Huang, H.-J. (2002). Transcriptional Regulation of a Rice Mitogen-Activated Protein Kinase Gene, *OsMAPK4*, in Response to Environmental Stresses. Plant Cell Physiology **43**: 958-963.

Gapper, C. and Dolan, L. (2006). Control of Plant Development by Reactive Oxygen Species. Plant Physiol. **141**: 341-345.

Groppa, M. D. and Benavides, M. P. (2007). Polyamines and abiotic stress: recent advances. Amino Acids **Epub ahead of print**.

Guo, H.-S., Xie, Q., Fei, J.-F. and Chua, N.-H. (2005). MicroRNA Directs mRNA Cleavage of the Transcription Factor NAC1 to Downregulate Auxin Signals for *Arabidopsis* Lateral Root Development. The Plant Cell **17**: 1376-1386.

Halliwell, B. (2006). Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. Plant Physiol. **141**: 312-322.

Harper, J. F., Ghislain, B. and Harmon, A. (2004). Decoding Ca2+ Signals Through Plant Protien Kinases. Annual Review of Plant Biology **55**: 263-288.

He, C., Fong, S. H. T., Yang, D. and Wang, G.-L. (1999). BWMK1, a Novel MAP Kinase Induced by Fungal Infection and Mechanical Wounding in Rice. Molecular Plant-Microbe Interactions **12**: 1064-1073.

He, X.-J., Mu, R.-L., Cao, W.-H., Zhang, Z.-G., Zhang, J.-S. and Chen, S.-Y. (2005). AtNAC, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. The Plant Journal **44**: 903-916.

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Kreis, M., Zhang, S., Hirt, H., Wilson, C., Heberle-Bors, E., Ellis, B. E., Morris, P. C., Innes, R. W., Ecker, J. R., Scheel, D., Klessig, D. F., Machida, Y., Mundy, J., Ohashi, Y. and Walker, J. C. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends in Plant Science **7**: 301-308.

Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U. and Speed, T. P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4**: 249-264.

Jin, H., Axtell, M. J., Dahlbeck, D., Ekwenna, O., Zhang, S., Staskawicz, B. and Baker, B. (2002). NPK1, an MEKK1-like Mitogen-Activated Protein Kinase Kinase Kinase, Regulates Innate Immunity and Development in Plants. Developmental Cell **3**: 291-297.

Jonak, C., Nakagami, H. and Hirt, H. (2004). Heavy Metal Stress. Activation of Distinct Mitogen-Activated Protein Kinase Pathways by Copper and Cadmium. Plant Physiology **136**: 3276-3283.

Jouannic, S., Hamal, A., Leprince, A.-S., Tregear, J. W., Kreis, M. and Henry, Y. (1999). Plant MAP kinase kinase kinases structure, classification and evolution. Gene **233**: 1-11.

Kater, M. M., Dreni, L. and Colombo, L. (2006). Functional conservation of MADS-box factors controlling floral organ identity in rice and *Arabidopsis*. Journal of Experimental Botany **57**: 3433-3444.

Kim, J.-A., Agrawal, G. K., Rakwal, R., Han, K.-S., Kim, K.-N., Yun, C.-H., Heu, S., Park, S.-Y., Lee, Y.-H. and Jwa, N.-S. (2003). Molecular cloning and mRNA expression analysis of a novel rice (Oryza sativa L.) MAPK kinase kinase, *OsEDR1*, and ortholog of *Arabidopsis AtEDR1*, reveal its role in defense/stress signalling pathways and development. Biochemical and Biophysical Research Communications **300**: 868-876.

Kovtun, Y., Chiu, W.-L., Tena, G. and Sheen, J. (2000). From the Cover: Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. PNAS **97**: 2940-2945.

Kovtun, Y., Chiu, W.-L., Zeng, W. and Sheen, J. (1998). Supression of auxin signal transduction by a MAPK cascade in higher plants. Nature **395**: 716-720.

Krysan, P. J., Jester, P. J., Gottwald, J. R. and Sussman, M. R. (2002). An *Arabidopsis* Mitogen-Activated Protein Kinase Kinase Kinase Gene Family Encodes Essential Postive Regulators of Cytokinesis. The Plant Cell **14**: 1109-1120.

Kwak, J. M., Nguyen, V. and Schroeder, J. I. (2006). The Role of Reactive Oxygen Species in Hormonal Responses. Plant Physiol. **141**: 323-329.

Lieberherr, D., Thao, N. P., Nakashima, A., Umemura, K., Kawasaki, T. and Shimamoto, K. (2005). A Sphingolipid Elicitor-Inducible Mitogen-Activated Protien Kinase Is Regulated by the Small GTPase OsRac1 and Heterotrimeric G-Protein in Rice. Plant Physiol. **138**: 1644-1652.

Lombaerts, M., Peltola, P. H., Visse, R., den Dulk, H., Brandsma, J. A. and Brouwer, J. (1999). Characterization of the *rhp7+* and *rhp16+* genes in Schizosaccharomyces pombe. Nucleic Acids Research **27**: 3410-3416.

Ma, S., Gong, Q. and Bohnert, H. J. (2006). Dissecting salt stress pathways. Journal of Experimental Botany **57**: 1097-1107.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science **7**: 405-410.

Munns, R. (2005). Genes and salt tolerance: bringing them together. New Phytologist **167**: 645-663.

Nakagami, H., Pitzschke, A. and Hirt, H. (2005). Emerging MAP kinase pathways in plant stress signalling. Trends in Plant Science **10**: 339-346.

Nakashima, M., Hirano, K., Nakashima, S., Banno, H., Nishihama, R. and Machida, Y. (1998). The Expression Pattern of the Gene for NPK1 Protein Kinase Related to Mitogen-Activated Protein Kinase Kinase Kinase (MAPKKK) in a Tobacco Plant: Correlation with Cell Proliferation. Plant Cell Physiol. **39**: 690-700.

Nishihama, R., Soyano, T., Ishikawa, M., Araki, S., Tanaka, H., Asada, T., Irie, K., Ito, M., Terada, M., Banno, H., Yamazaki, Y. and Machida, Y. (2002). Expansion of the Cell Plate in Protein Cytokinesis Requires a Kinesin-like Protein/MAPKKK Complex. Cell **109**: 87-99.

Patterson, J., Ford, K., Cassin, A., Natera, S. and Bacic, A. (2007). Increased Abundance of Proteins Involved in Phytosiderophore Production in Boron-Tolerant Barley. Plant Physiol. **144**: 1612-1631.

Pitzschke, A. and Hirt, H. (2006). Mitogen-Activated Protein Kinases and Reactive Oxygen Species Signaling in Plants. Plant Physiol. **141**: 351-356.

Pretty, J. (2007). Agricultural sustainability: concepts, principles and evidence. Phil. Trans. R. Soc. Lond. **Epub ahead of print**.

Protocol, E. C. Event-specific Method for the Quantification of Rice Line LLRICE62 Using Real-time PCR.

Ralph, S., Park, J.-Y., Bohlmann, J. and Mansfield, S. D. (2006). Dirigent proteins in conifer defense: gene discovery, phylogeny, and differential wound- and insect-

induced expression of a family of *DIR* and *DIR-like* genes in spruce (Picea spp.). Plant Molecular Biology **60**: 21-40.

Ray, S., Agarwal, P., Arora, R., Kapoor, S. and Tyagi, A. K. (2007). Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (*Oryza sativa* L. ssp. indica). Mol Genet Genomics **278**: 493-505.

Ren, D., Yang, H. and Zhang, S. (2002). Cell Death Mediated by MAPK Is Associated with Hydrogen Peroxide Production in *Arabidopsis*. TheJournal of Biological Chemistry **277**: 559-565.

Reyna, N. S. and Yang, Y. (2006). Molecular Analysis of the Rice MAP Kinase Gene Family in Relation to *Magnaporthe grisea* Infection. Molecular Plant-Microbe Interactions **19**: 530-540.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000). Over-expression of a single Ca2+-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. The Plant Journal **23**: 319-327.

Sasabe, M., Takeuchi, K., Kamoun, S., Ichinose, Y., Govers, F., Toyoda, K., Shiraishi, T. and Yamada, T. (2000). Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture. European Journal of Biochemistry **267**: 5005-5013.

Seki, M., Kamei, A., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2003). Molecular responses to drought, salinity and frost: common and different paths for plant protection. Current Opinion in Biotechnology **14**: 194-199.

Shou, H., Bordallo, P., Fan, J.-B., Yeakley, J. M., Bibikova, M., Sheen, J. and Wang, K. (2004a). Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc. Natl. Acad. Sci. USA **101**: 3298-3303.

Shou, H., Bordallo, P. and Wang, K. (2004b). Expression of the *Nicotiana* protein kinase (NPK1) enhanced drought tolerance in transgenic maize. Journal of Experimental Botany **55**: 1013-1019.

Souer, E., van Houwelingen, A., Kloos, D., Mol, J. and Koes, R. (1996). The *No Apical Meristem* Gene of Petunia Is Required for Pattern Formation in Embryos and Flowers and Is Expressed at Meristem and Primordia Boundaries. Cell **85**: 159-170.

Song, F. and Goodman, R. M. (2002). *OsBIMK1*, a rice MAP kinase gene involved in disease resistance responses. Planta **215**: 997-1005.

Song, D., Chen, J., Song, F. and Zhang, Z. (2006). A Novel Rice MAPK Gene, *OsBIMK2*, is Involved in Disease-Resistance Responses. Plant Biol. **8**: 587-598.

Soyano, T., Nishihama, R., Morikiyo, K., Ishikawa, M. and Machida, Y. (2003). NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes & Development **17**: 1055-1067.

Sperotto, R. A., Boff, T., Duarte, G. L. and Fett, J. P. (2007). Increased senescence-associated gene expression and lipid peroxidation induced by iron deficiency in rice roots. Plant Cell Rep **Epub ahead of print**.

Storey, J. D., and Tibshirani, R. (2003). Statistical significance for genomewide studies. Proceedings of the National Academy of Sciences **100**: 9440-9445.

Takayama, S. and Isogai, A. (2003). Molecular mechanisms of self-recognition in *Brassica* self-incompatibility. Journal of Experimental Botany **54**: 149-156.

Takken, F. L. W., Albrecht, M. and Tameling, W. I. L. (2006). Resistance protiens: molecular switches of plant defence. Current Opinion in Plant Biology **9**: 383-390.

Teige, M., Scheikl, E., Eulgem, T., Doczi, R., Ichimura, K., Shinozaki, K., Dangl, J. L. and Hirt, H. (2004). The MKK2 Pathway Mediates Cold and Salt Stress Signaling in *Arabidopsis*. Molecular Cell **15**: 141-152.

Ueda, A., Kathiresan, A., Bennett, J. and Takabe, T. (2006). Comparative transcriptome analyses of barley and rice under salt stress. Theor. Appl. Genet. **Epub ahead of print.**

Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006). Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Current Opinion in Biotechnology **17**: 113-122.

Vinocur, B. and Altman, A. (2005). Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Current Opinion in Biotechnology **16**: 123-132.

Walker, J. C. and Zhang, R. (1990). Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of *Brassica*. Nature **345**: 743-746.

Walz, C., Giavalisco, P., Schad, M., Juenger, M., Klose, J. and Kehr, J. (2004). Proteomics of curcurbit phloem exudate reveals a network of defence protiens. Phytochemistry **65**: 1795-1804.

Wan, B., Lin, Y. and Mou, T. (2007). Expression of rice Ca2+-dependent protein kinases (CDPKs) genes under different environmental stresses. FEBS Letters **581**: 1179-1189.

Wen, J.-Q., Oono, K. and Imai, R. (2002). Two Novel Mitogen-Activated Protein Signaling Components, OsMEK1 and OsMAP1, Are Involved in a Moderate Low-Temperature Signaling Pathway in Rice. Plant Physiol. **129**: 1880-1891.

Xiong, L., Schumaker, K. S. and Zhu, J.-K. (2002). Cell Signaling during Cold, Drought, and Salt Stress. The Plant Cell **Supplement 2002**: S165-S183.

Xiong, L. and Yang, Y. (2003). Disease Resistance and Abiotic Stress Tolerace in Rice Are Inversely Modulated by an Abscisic Acid-Inducible Mitogen-Activated Protein Kinase. The Plant Cell **15**: 745-759.

Yamanouchi, U., Yano, M., Lin, H., Ashikari, M. and Yamada, K. (2002). A rice spotted leaf gene, *Spl7*, encodes a heat stress transcription factor proten. Proc. Natl. Acad. Sci. USA **99**: 7530-7535.

Yang, G. and Komatsu, S. (2000). Involvement of Calcium-Dependent Protien Kinase in Rice (*Oryza sativa* L.) Lamina Inclination Caused by Brassinolide. Plant Cell Physiol. **41**: 1243-1250.

Yang, J., Zhang, J., Liu, K., Wang, Z. and Lijun, L. (2007). Involvement of polyamines in the drought resistance of rice. Journal of Experimental Botany **Epub ahead of print**.

Yeh, C.-M., Hsiao, L.-J. and Huang, H.-J. (2004). Cadmium Activates a Mitogen-Activated Protein Kinase Gene and MBP Kinases in Rice. Plant Cell Physiol. **45**: 1306-1312.

Yoshioka, H., Numata, N., Nakajima, K., Katou, S., Kawakita, K., Rowland, O., Jones, J. D. G. and Doke, N. (2003). *Nicotiana benthamiana* gp91phox Homologs NbrbohA and NbrbohB Participate in H_2O_2 Accumulation and Resistance to *Phytophthora infestans*. The Plant Cell **15**: 796-718.

Zhang, A., Jiang, M., Zhang, J., Tan, M. and Hu, X. (2006). Mitogen-Activated Protein Kinase Is Involved in Abscisic Acid-Induced Antioxidant Defense and Acts downstream of Reactive Oxygen Species Production in Leaves of Maize Plants. Plant Physiol. **141**: 475-487.

Zhang, J. Z., Creelman, R. A. and Zhu, J.-K. (2004). From Laboratory to Field. Using Information from *Arabidopsis* to Engineer Salt, Cold, and Drought Tolerance in Crops. Plant Physiol. **135**: 615-621.

Zhao, Y., Dai, X., Blackwell, H. E., Schreiber, S. L. and Chory, J. (2007). SIR1, an Upstream Component in Auxin Signaling Identified by Chemical Genetics. Science **301**: 1107-1110.

Zhu, C. and Perry, S. E. (2005). Control of expression and autoregulation of AGL15, a member of the MADS-box family. The Plant Journal **41**: 583-594.

Zimmermann, R. and Werr, W. (2005). Pattern formation in the monocot embryo as revealed by NAM and CUC3 orthologues from Zea mays L. Plant Molecular Biology **58**: 669-685.

Gene name	Public ID	Forward and Reverse primer sequences
Phospholipase D (Endogenous control)	CAD11899	OsPLD-f: 5'- TGG TGA GCG TTT TGC AGT CT -3'; OsPLD-r: 5'- CTG ATC CAC TAG CAG GAG GTC C -3';
MPK1 (also characterized as OsMAPK6[1]; OsSIPK[2])	AB183398	MPK1-f: 5'- CGA CAG TGA TCA CTA GTT GT -3'; MPK1r: 5- GCA GAA TTT AGG CCG TCA TC -3';
MPK2	BAC99508	MPK2-f: 5'- GAA GAA CGG AAG CAG ATC AC -3'; MPK2-r: 5'- GTC CGA ATT TGC GAT GTC AG -3';
MPK3 (also described as OsMAP3[3])	AAG40591	MPK3-f: 5'- CGA TGA TCT TCA ACT GTC CC -3'; MPK3-r: 5'- CAT ACA CCA AGT AAT GGT CC -3';
MPK4 (also named OsMSRMK3[4], OsMAPK4[5])	CAB61889	MPK4-f: 5'- CTG TGG ACC TCT ACT TGT AG -3'; MPK4-r: 5'- TCT CAG CAA GTC AGC ATA GC -3';
MPK5 (also published under the name OsMAPK2[6], OsMSRMK2[7], OsMAP1[8], OsBIMK1[9], OsMAPK5[10])	AF479883	MPK5-f: 5'- ACG AGG ACC AAA TGA AGC AGC -3'; MPK5-r: 5'- AGC AGC CAC AAC TTG CAG AGA -3';
MPK6	NM_197522	MPK6-f: 5'- GTC AGA CTA CTC TGA TAG GC -3'; MPK6 r: 5'- GAG CAC TCC TCG TAA AGT AG -3';
MPK7	AK099472	MPK7-f: 5'- TCA TCT GGA GGG AAT CCT TG -3'; MPK7 r: 5'- TGC TCA TTC GCC CAA CAA GT -3';
MPK8 (also known as OsWJUMK1[11])	AJ512643	MPK8-f: 5'- GTC AGG CAG TTG TGG AAT TG -3'; MPK8 r: 5'- CTC CTT TCT TCA ACC TTC GC -3';
MPK9	AAT44204	MPK9-f: 5'- GAT TCC AGG TAG AAC AGG AC -3'; MPK9-r: 5'- GCT CTC TTT CTG AGC TAT CC -3';
MPK10	NM_192924	MPK10-f: 5'- GTG GCT CAG CAA TTC CAA TG -3'; MPK10-r: 5'- TGC CAC TTG TGA AAC CTG AG -3';
MPK11	BAD69155	MPK11-f: 5'- TCA GGT CTA CTA TTG TTC AC -3'; MPK11-r: 5'- GCA ATA CTG GAC CAA CAA CTC -3';
MPK12 (also characterized as OsBWMK1[12])	AF177392	MPK12-f: 5'- TCC AAG TAC ACA CCA GCT ATT G -3'; MPK12-r: 5'- ATA GCA TCT AAG GAG GGT GTT C -3';
MPK13 (also named OsBIMK2[13])	AY524973	MPK13-f: 5'- TGG AGT ATC ACC CAC AGA TG -3'; MPK13-r: 5'- CTG TAG TTC TCC TCA AGG TG -3';
MPK14	AAS98446	MPK14-f: 5'- AGA ATG GTA TCT CTG AGG AC -3'; MPK14-r: 5'- AGA GCC TAG AGT ATC AGC TC -3';
MPK15	ABA92667	MPK15-f: 5'- ACA GCC AGT GTC ACT GAG GAT C -3'; MPK15-r: 5'- GAA CAG TTA CCG TAA CGC AG -3';
MPK16	NM_192298	MPK16-f: 5'- ACC CAC AGC AGA AGC TTA CA -3'; MPK16-r: 5'- AGA CGA TGT GAA GTC ACC AG -3';
MPK17	AAT39148	MPK17-f: 5'- AGT ACC ATC CTC AGA TGA TGC -3'; MPK17-r: 5'- GTC CAT CTG TCA CTG ATG AG -3';

Table 4.1. Sequences of primers used for qRTPCR of OsMAPK genes. Several of the genes have multiple genbank entries and have been characterized under more than one designation. The names given in the table are those given by Reyna and Yang (2006), the source of the qRT-PCR primers.

[1]OsMAPK6 (Lieberherr *et al.* 2005); [2]OsSIPK (AJ535841); [3]OsMAP3; [4]OsMSRMK3 (AJ512642) (Agrawal *et al.* 2003a,b); [5]OsMAPK4 (AJ251330) (Yeh *et al.* 2004; Fu *et al*. 2002; Agrawal *et al*. 2003b); [6]OsMAPK2 (AJ250331) (Agrawal *et al*. 2003b; Yeh *et al*. 2004); [7]OsMSRMK2 (AJ486975) (Agrawal *et al*. 2002); [8]OsMAP1 (AF216315) (Wen *et al.* 2002; Agrawal *et al*. 2003b); [9]OsBIMK1 (AF332873) (Song and Goodman 2002; Agrawal *et al*. 2003b) or [10]OsMAPK5 (Xiong and Yang 2003); [11]OsWJUMK1 (Agrawal *et al.* 2003a,b); [12]OsBWMK1 (Cheong *et al.* 2003; He *et al*. 1999; Agrawal *et al.* 2003a,b); [13]OsBIMK2 (Song *et al.* 2006).

Genes Downregulated in caNPK1 Transgenic Plants

Short ID	Expression by RMA	Expression by MAS5.0	Affymetrix ID	Public ID	Annotation ID	E-score	ID
Floral/reproductive							
NAM (no apical meristem) protein-like, O.sativa.	-2.95	-2.28	Os.17912.2.S1_x_at	AK066107.1	BAD53136.1	4E-122	100%
S-locus glycoprotein family, putative	-1.65	-2.07	OsAffx.14131.1.S1_at	9632.m03298			
MADS57	-1.58	-1.56	Os.26399.1.S1_at	AY177702.1			
Metabolism							
Phospholipid/glycerol acyltransferase-like	-1.77	-1.67	Os.10477.2.S1_x_at	AK108343.1	BAD28269.1	6E-05	66%
Putative ACT-domain containing protein	-1.84	-1.91	Os.53355.1.S1_s_at	AK100703.1	BAD15455.1	0E+00	99%
Protease							
Serine carboxypeptidase	-2.13	-5.76	Os.50502.1.S1_at	AK121476.1	ABG22477.1	0E+00	75%
Kinase							
CDPK11	-1.89	-1.90	Os.14684.1.S1_at	AK065509.1			
Hypothetical protein/CDPK-related	-1.55	-1.60	Os.54732.1.S1_at	AK106629.1	NP_565281.1	4E-102	61%
Stress response							
Putative dehydration-responsive protein	-1.54	-1.75	Os.28427.1.S2_a_at	AK104585.1	AAT47027.1	3E-129	100%
Transcription factor							
RF-A containing putative DNA-binding protien	-1.96	-1.62	Os.53868.1.S1_at	AK100505.1	BAD87224.1	5E-49	64%
RF-A containing putative DNA-binding protein	-1.99	-2.18	Os.53868.1.S1_s_at	AK100505.1		5E-49	64%
Translation/protein synthesis							
Hypothetical protein/RNA-binding protein	-1.79	-1.91	Os.21875.1.S1_at	CA757906		3E-47	99%
Putative splicing factor	-1.42	-1.65	Os.50814.2.S1_at	AK071377.1	NP_201232.1	0E+00	96%
Reverse transcriptase	-2.26	-2.13	Os.54381.1.S1_at				

Table 4.2. Genes downregulated in caNPK1 transgenic plants. Genes in this genelist have at least 1.5-fold greater expression in Nipponbare wild type than caNPK1 transgenics. Differentially expressed unknown proteins were excluded from this list but appear in Appendix C. A MADS-box transcription factor and a putative MADS-box transcription factor contribute to floral and reproductive structure development, the major functional category that appears to be downregulated by the presence of caNPK1. The public sequence ID (given by Affymetrix) and the sequence used to provide annotation is given if annotation was arrived at through BlastN or BlastP search.

Genes Upregulated in caNPK1 Transgenic Plants

Short ID	Expression by RMA	Expression by MAS5.0	Affymetrix ID	Public ID	Annotation ID	E-score	ID
Bar gene							
Bar gene	84.94	245.47	RPTR-Os-X17220-1_at				
Protease							
Unknown/similar to Peptidase	1.55	1.53	Os.5792.1.S1_a_at	AK120820.1			
Subtilisin	1.15	2.29	OsAffx.3233.1.S1_at	9631.m01328			
Kinase							
Putative wall-associated protein kinase	6.11	4.68	Os.21240.2.S1_at	BQ908738	AAP52081.1	0E+00	91%
Protein kinase-like domain	1.93	1.95	Os.19044.1.S1_at	CA764426	NM_001070648.1	3E-165	99%
Receptor-like protein kinase, O. sativa	1.61	1.64	Os.2436.1.S1_at	AK102893.1	AAF34428.1	0E+00	97%
Unknown/potential kinase domain	1.55	1.52	Os.54531.1.S1_at				
Methionine biosynthesis							
S-adenosylmethionine synthetase 1	2.10	2.58	Os.16099.1.S1_at	CR286816	NM_001049329	8E-82	94%
Putative Aci-reductone dioxygenase	10.61	8.22	Os.4766.1.S1_at	AK103834.1	ABB47627.1	8E-102	100%
Stress response							
Putative MAWD binding protein	1.68	1.62	Os.20404.1.S1_at	AK101084.1	BAA88529.1	1E-156	100%
Lipase-like	2.09	2.92	Os.37457.1.S1_at	AK058442.1	BAC16480.1	0E+00	100%
Cytochrome P450 family protein	1.88	1.84	Os.34992.1.S1_at	AK069394.1	ABF96180.1	1E-144	100%
Putative salt tolerance protein	1.78	1.73	Os.27553.2.S1_at	AK063477.1	BAC57372.1	9E-141	100%
Putative dirigent protein	8.56	6.96	Os.12381.1.S1_s_at	AK106022.1	BAC9525.1	2E-103	100%
Putative dirigent protein	11.36	9.45	Os.12381.1.S1_x_at	AK106022.1	BAC9525.1	2E-103	100%
Spl7b	9.57	8.11	Os.20851.1.A1_at	AK064111.1			
Spl7b	5.33	7.19	Os.20851.1.A1_x_at	AK064111.1			
Rhodanese-like domain containing protein	2.46	2.70	Os.34982.1.A1_at	AK119900.1	NM_001058845.1	9E-109	100%
Rhodanese-like domain containing protein	2.26	2.35	Os.34982.1.A1_x_at	AK119900.1	NM_001058845.1	9E-109	100%
Putative far-red impaired response protein	1.64	2.27	Os.35093.1.A1_x_at	NM_188076.1	BAD36174.1	0E+00	78%
Putative DNA repair protien rhp16	2.50	2.06	Os.50017.1.S1_at	AK119665.1	BAD31171.1	7.0E-52	100%
NB-ARC domain, putative	1.63	3.12	OsAffx.15443.1.S1_at	9634.m01581			
Biotin biosynthesis							
Putative 8-amino-7-oxononanoate synthase	1.78	1.58	Os.25227.1.S1_s_at	AK069723.1	BAD87813.1	0E+00	100%
Putative adenosylmethionine-8-amino-7-oxononanoate aminotransferase	1.61	1.57	Os.9720.1.S1_at	AK100945.1	BAD05190.1	0E+00	100%
Putative 8-amino-7-oxononanoate synthase	1.47	1.55	Os.25227.1.S1_x_at	AK069723.1	BAD87813.1	0E+00	100%
Starch biosynthesis							
Beta-glucosidase	1.90	1.54	OsAffx.15538.1.S1_at	9634.m02112			
Serine biosynthesis							
Putative cysteine synthase	1.32	5.64	Os.53027.1.S1_at	AK070508.1	BAD53767.1	0E+00	100%
Protein binding							
Unknown protein/F-box/LRR-repeat	1.83	2.17	Os.50765.1.S1_at	AK058412.1	ABF94707.1	8E-13	27%
Leucine rich repeat, putative	2.03	4.02	OsAffx.31316.1.S1_at	9639.m03229			
Putative leucine rich repeat-containing protein	1.82	1.93	OsAffx.31705.2.S1_at	9640.m01111			
Protein modification							
Glycosyl transferase family 17 protein, putative	1.94	1.71	Os.33273.1.S1_at	AK101128.1	ABA99377.1	0E+00	100%
Hormone biosynthesis							
Potential match to putative giberellin regulated protein	1.54	1.64	Os.17900.1.S1_s_at	BI803310	AAT47046.1	4E-30	98%
Shikimate pathway							
Potential match to 3-deoxy-D-arabino heptulosonate-7-phosphate synthase	1.48	1.52	Os.20183.1.S1_at	AU175102	AB122083.1		
Signal transduction							
Putative steroleosin/oxidoreductase	1.70	2.33	Os.49976.1.S1_at	AK119471.1	BAD23084.1	0E+00	100%
Transcription factor							
Leucine zipper protein-like	1.68	1.66	Os.52715.1.S1_at	AK068895.1	BAD28478.1	0E+00	99%
SNF2P putative	5.11	3.79	Os.7306.1.S1_at	AK066228.1	ABF93482.1	0E+00	100%
Translation/protein synthesis							
Ribosomal protein-like	84.54	110.77	Os.31233.1.S1_at	AK107646.1	BAD88049.1	0E+00	99%
Rpp14 family, putative	1.64	4.61	OsAffx.20707.5.S1_at	9633.m01690			
Transposon							
Putative TNP-like transposable elements	1.83	5.05	Os.48948.1.S1_x_at	NM_196441.1			
Unknown/ possible match to CRR3 putative polyprotein gene	4.41	3.85	Os.52004.1.S1_at	AK064520.1	DQ458292.1	2E-158	100%

Table 4.3. Genes upregulated in caNPK1 transgenic plants. Genes in this genelist have at least 1.5-fold greater expression in caNPK1 transgenics than Nipponbare wild type. Differentially expressed unknown proteins were excluded from this list but appear in Appendix C. Numerous stress related genes appear to be upregulated by the presence of caNPK1. In addition, kinases upregulated in caNPK1-expressing plants are categorized in the protein kinase class, as opposed to specifically the calcium-dependent protien kinase class, two members of which are potentially downregulated by the presence of caNPK1 (Table 4.2.)

Gene name	Affymetrix ID	Public ID	Regulation	Primer sequences
CDPK11	Os.14684.1.S1_at	AK065509	↓	CDPK11-f: 5'-GCCAACAGAATCGAGACCATATCC-3' CDPK11-r: 5'-TTTGTAACCACAACACTTCACACA-3'
putative dehydration-responsive protein	Os.28427.1.S2_a_at	AK104585	↓	DRP-f: 5'-CTCAGCTTCGAACCATAAGGAGGA-3' DRP-r: 5'-TGAAGTGGAGCGTCATCTTGGA-3'
putative wall-associated protein kinase	Os.21240.2.S1_at	BQ908738	↑	WAPK-f: 5'-GCCGAACCACTGGTGATGCTTAAA-3' WAPK-r: 5'-CCTCATAAATCTTCTGGTGTCAAGC-3'
S-adenosylmethionine synthetase 1	Os.16099.1.S1_at	CR286816	↑	SAMS1-f: 5'-GACACCAGATTTGGACAGAAGTGC-3' SAMS1-r: 5'-AGAAGCTTGTGTGGTGAGTGGTGA-3'
potential match to 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase	Os.20183.1.S1_at	AU175102	↑	AU175102-f: 5'-CGTGTCTCACCGGGATTTATTCCCAA-3' AU175102-r: 5'-AGCCACCTAAGAACCTTGTGTGTG-3'
R2R3Myb protein gene	Os.19044.1.S1_at	CA764426	↑	R2R3-f: 5'- AGA ACT TCT CCT TGT ACG GTG AGC -3' R2R3-r: 5'- GCA ATG GAG CAG CTG GAG ATG AAT -3'
putative salt tolerance protein	Os.27553.2.S1_at	AK063477	↑	STP-f: 5'- AAC TCA CAT GCG AAG GTT TGA GGC -3' STP-r: 5'- TCT TGA GCC AAC CTT TCT CGA CCA -3'
putative dirigent protein	Os.12381.1.S1_s_at	AK106022	↑	DIR-f: 5'- CAT GGA AGG AGA TTC GTC AGG AGT -3' DIR-r: 5'- AGT TGG AGG TGC TGT TGC TGT AGT -3'
Rhodanese-like domain containing protein	Os.34982.1.A1_at	AK119900	↑	Rhod-f: 5'- TGT GGG TTG CCT AAG TGG AGT AAG -3' Rhod-r: 5'- TTT CCA CCC ATG CCA TGT AAC CTC -3'

Table 4.4. Primer sequences of genes selected for confirmation by qRT-PCR.

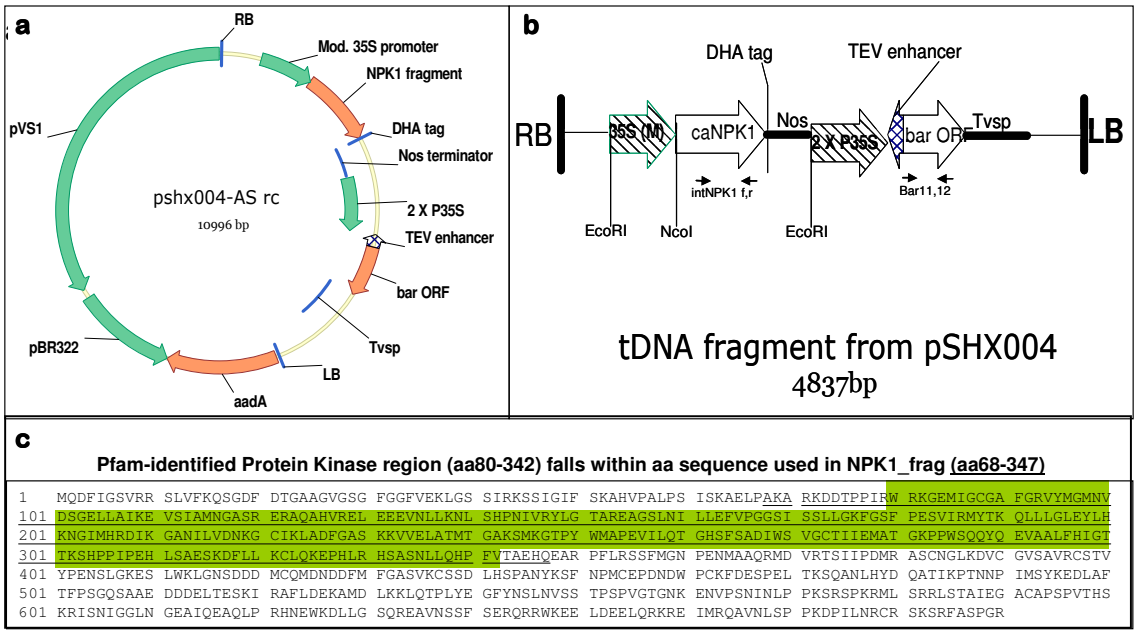

Figure 4.1. Construct used for transformation of Nipponbare rice. Plasmid map of pSHX004, a dual host vector (a). Map of tDNA fragment from pSHX004 showing 1.8kb EcoRI dropout and locations of primers for 604bp caNPK1 specific PCR product and 520bp Bar specific PCR product (b). Kinase domain (aa80-342) and caNPK1 coding domain translation (aa68-347) indicated on protein sequence of full-length tobacco NPK1 (BAA05648) (c).

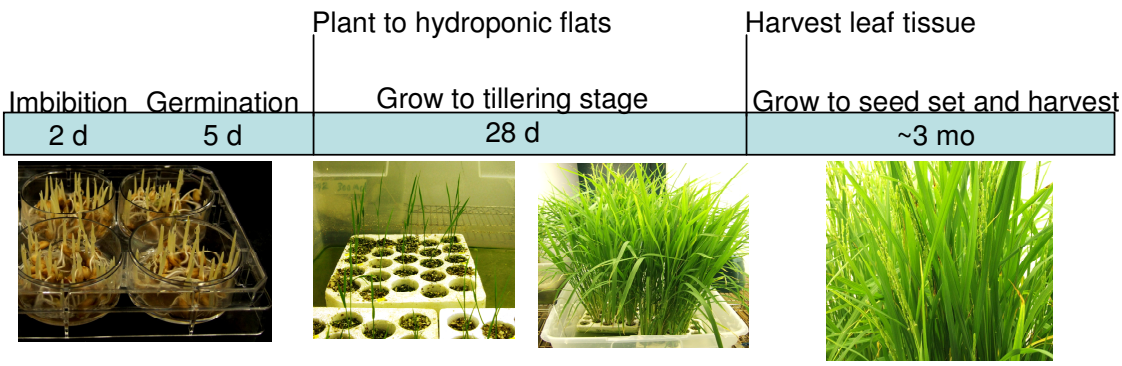

Figure 4.2. Hydroponic culture of rice. Significant time points in the culture include a total of seven days of germination, two days immersed in distilled water for seed imbibitions, then then up to 25 seeds are arranged in a single layer in six-well tissue culture plates and allowed to germinate on moistened paper towel or Whatmann paper. Plants begin to tiller on average 4 weeks after seedings are transplanted to the styrofoam racks that serve as hydroponic flats. Plastic pet screen attached to the bottom of the rack prevents the seedling and vermiculite from falling through. Vermiculite ensures that the seedling is in a moist but not flooded environment during early growth and provides some support for the plant prior to tillering. Following the harvest of a single leaf, the plant is kept in solution until flowering and seed set.

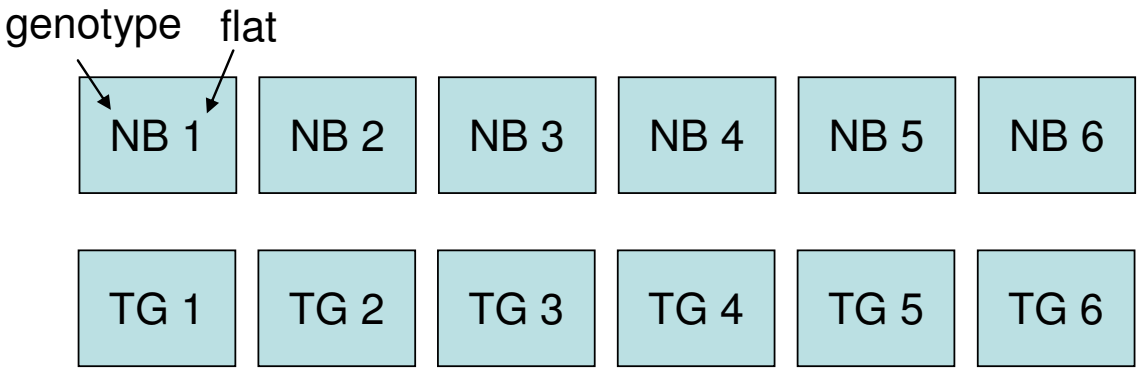

Figure 4.3: Experimental layout. Each rectangle represents one Affymetrix Rice Genome Array chip. Samples applied to each chip are designated by genotype and flat of sample origin (NB=Nipponbare wild type, TG=PCR positive 178.1). Samples consist of equal amounts of RNA from four plants of confirmed genotype grown within the same flat. Proc mixed analysis (SAS) was set to use genotype as the fixed effect and flat as a random effect.

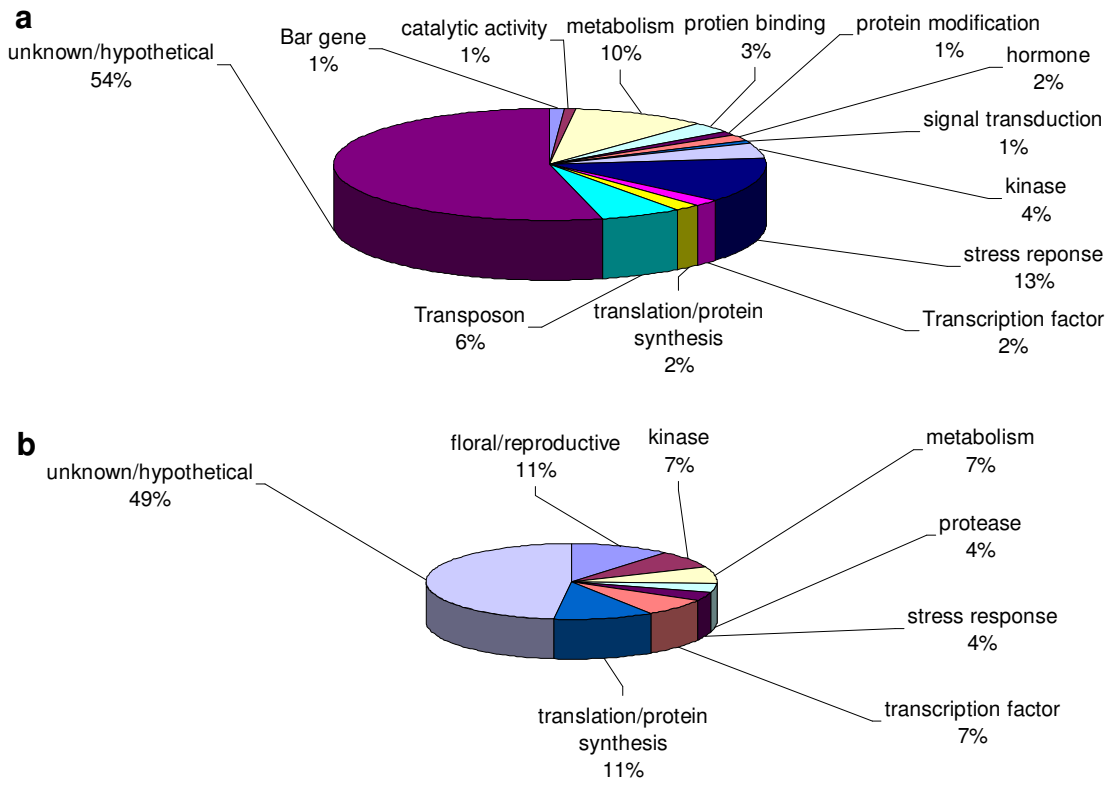

Figure 4.4. Genes with greater than 1.5-fold differential expression. Genes upregulated in caNPK1 transgenics (a). Functional characterizations of 99 genes with higher levels of expression in in caNPK1 transgenic plants can be grouped into 12 groups. Downregulated genes (b). Downregulated genes could be grouped into 8 functional categories. The majority of upregulated and downregulated transcripts correspond with unknown or hypothetical proteins. The highest percentage of transcripts in a functional category in downregulated and upregulated gene lists are related to flowering and reproduction (3 genes out of a total of 27) and stress response (13 out of 99), respectively.

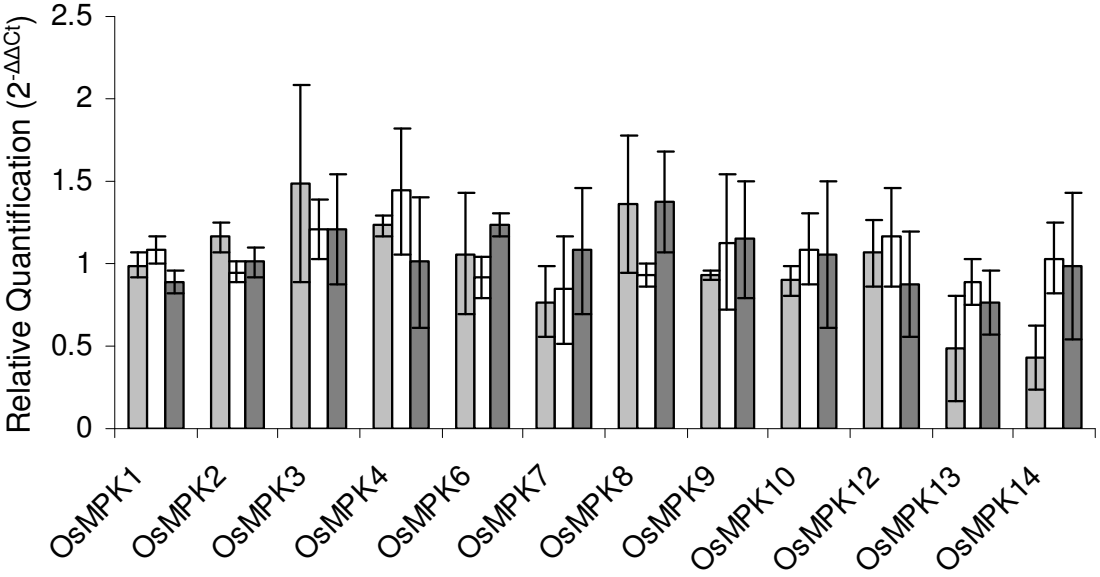

Figure 4.5. Relative quantification of selected OsMPK gene expression. Gene expression in each sample is determined by ΔΔCt relative to one Nipponbare sample (referred to as calibrator). Averages of fold-difference from the calibrator are presented for R1S1 transgenic cDNA (▒), Nipponbare samples (□) and caNPK1 transgenic samples (■). OsMPK genes with good amplification and consistent relative quantification among Nipponbare samples are shown.

Chapter 5:

Genetic engineering approaches to improve bioethanol production from maize

François Torney, Lorena Moeller, Andréa Scarpa and Kan Wang

ELSEVIER

Genetic engineering approaches to improve bioethanol production from maize

François Torney, Lorena Moeller, Andréa Scarpa and Kan Wang

Biofuels such as bioethanol are becoming a viable alternative to fossil fuels. Utilizing agricultural biomass for the production of biofuel has drawn much interest in many science and engineering disciplines. As one of the major crops, maize offers promise in this regard. Compared to other crops with biofuel potential, maize can provide both starch (seed) and cellulosic (stover) material for bioethanol production. However, the combination of food, feed and fuel in one crop, although appealing, raises concerns related to the land delineation and distribution of maize grown for energy versus food and feed. To avoid this dilemma, the conversion of maize biomass into bioethanol must be improved. Conventional breeding, molecular marker assisted breeding and genetic engineering have already had, and will continue to have, important roles in maize improvement. The rapidly expanding information from genomics and genetics combined with improved genetic engineering technologies offer a wide range of possibilities for enhanced bioethanol production from maize.

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Introduction

The world energy demand is increasing steadily as the human population grows and economic development progresses. However, the current predominant energy source — the fossil fuel supply — is limited. This emphasizes the need to complement fossil-fuel-based energy sources with renewable energy sources, such as agricultural biomass (see Glossary) [1]. Maize, currently one of two major biofuel (see Glossary) crops in the United States, represents 31% of the world production of cereals and occupies a little over one fifth of the worldwide cereal-dedicated land [2]. In addition, maize is the second largest biotech crop (see Glossary) grown world wide, after soybean, and a little over 10% of its cultivated surface is dedicated to biotech varieties [3].

To date, most maize genetic engineering (see Glossary) has been performed using a few genotypes that are amenable to transformation and regeneration, but which do not always have the desired agronomic attributes [4,5] (see Figure 1). Improving our ability to introduce transgenes directly into inbred or elite genetic backgrounds is crucial for bioethanol production, because it reduces the time required for transgene introgression into elite maize lines. Other enabling technologies under development aim to improve the quality of transgene expression. These include tissue or developmental stage specific transgene expression, stringently regulated and induced gene expression [6••], site-specific integration of the transgenes [7], expression of multiple transgenes, and gene stacking (i.e. adding transgenes sequentially in a genome) [8,9].

The net energetic benefit of using maize, mainly its starch component [1], for bioethanol production has been extensively reviewed [10••,11] and is still debated among experts [11–13]. Our focus will be on the various possibilities that genetic engineering can offer to increase bioethanol production from maize (see Figure 2). This can be addressed from at least two angles: modifying biomass properties to reduce processing costs or increasing biomass yield (see Glossary) and reducing agricultural inputs. We will review the latest studies on maize biology related to these aspects. Promising work in other species that could lead to improved bioethanol production in maize will also be discussed.

Genetic engineering to modify biomass properties

Two key parts of maize plants can be converted into bioethanol: the kernel, which is mainly made of starch, and the stover, which is predominantly made of lignin and cellulosic (cell wall) components. To convert them effectively into fermentable sugars for ethanol production, a range of approaches using genetic engineering have been explored. One strategy is to modify the characteristics and properties of starch or lignocellulose so that they can be converted more readily to the desired products. The other strategy is to introduce biomass conversion enzymes into plants so that they can aid the conversion process more effectively.

Starch composition

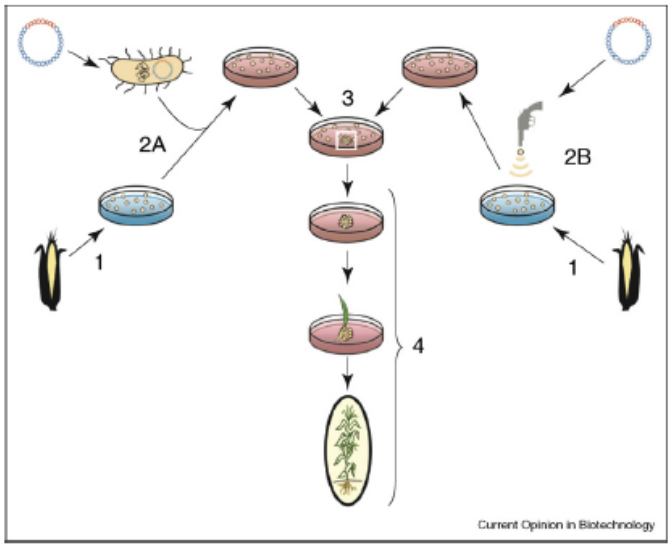
Today, ethanol from maize is produced almost exclusively from starch. The technologies and processes for

Glossary
Biofuel: Fuel produced from crop-derived carbohydrates. Includes bioethanol produced from fermentable sugars and biodiesel produced from plant oil
Biomass: Biological materials used for fuel or industrial production. Here, we refer to the sum of maize harvestable tissues
Biomass yield: Quantity of biomass per land surface unit
Biotech crops: Crops with enhanced agronomical or biological properties produced through genetic engineering
Effector gene: Gene coding for a protein involved directly in a physiological response process
Genetic engineering: A process involving the isolation, characterization and reintroduction of DNA into cells or organisms through recombinant DNA technology
Signal transduction components: Genes and their products involved in the relay of message between the stimulus perception and activation of effector genes
Sink strength: The ability of a sink organ (any organ, e.g. roots, developing seeds or immature leaves, that imports photosynthetic assimilates) to competitively mobilize assimilates toward itself

deriving ethanol from maize kernel starch have been well-established since the 1980s [13].

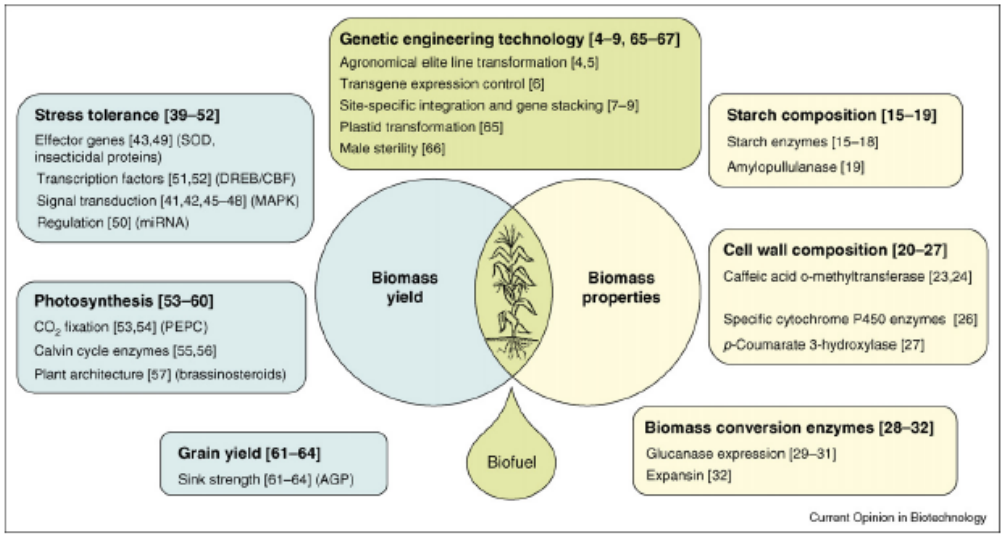
Advances in understanding the starch biosynthetic pathway have been reviewed elsewhere [14] and provide new ways to redesign starch for specific purposes [15,16]. Starch is composed of two glucose polymers, amylose and amylopectin. In amylose, glucose units are linked in a linear fashion by α1-4 linkages. Amylopectin, by contrast, is more branched and about 5% of its glucose units are linked by α1-6 linkages. Normal maize starches contain about 20–30% of amylose and 70–80% amylopectin. The amylose/amylopectin ratio in starch affects its physical and physicochemical properties, such as gelatinization and recrystallization [17]. Alteration in starch structure can be achieved by modifying genes encoding the enzymes responsible for starch synthesis, many of which have more than one isoform [15,18]. Transgenic lines with modified expression of specific starch synthases, starch branching enzymes or starch debranching enzymes are being generated in attempts to produce starch granules with increased or decreased crystallinity, and thus altered susceptibility to enzymatic digestion (M James, personal communication). Another strategy is to reduce the energy requirements for the starch to ethanol conversion process. For example, gelatinization is the first step in bioethanol production from starch. It is conceivable that a modified starch with decreased gelatinization temperature might require less energy for the conversion process. Recent research showed that expression of a

Figure 1



Two key approaches for the genetic transformation of maize. 1) Immature maize embryos are dissected from ears of corn harvested 11–14 days after pollination and placed on media containing nutrients and plant growth hormones (blue). The gene of interest can be introduced by one of two routes: 2A) the embryos are infected with an *Agrobacterium tumefasciens* strain that delivers the gene of interest and a selectable marker gene; or 2B) the embryos are bombarded with gold particles coated with one or more plasmids containing the gene of interest and a selectable marker gene. 3) Infected or bombarded embryos are placed on plant growth media supplemented with a selective agent (pink). Transformed cells expressing the selectable marker gene can proliferate and produce a callus mass (in square box). 4) The transgenic callus is cultured further and regenerated into mature transgenic maize plants that will subsequently be grown to maturity and analyzed.

Figure 2



Possible approaches to enhance biofuel production from maize biomass. Two main routes for enhancing maize bioethanol production through genetic engineering are reviewed here: a quantitative and qualitative approach. The first aims to increase the biomass production per land area (i.e. the biomass yield and its stability). The second aims to alter biomass properties and composition to generate conversion process-friendly products for ethanol production.

recombinant amylopullulanase in rice resulted in starch that when heated to 85 °C was completely converted into soluble sugars [19].

Cell wall composition

Maize stover (leaves and stalks) constitutes a large part of agricultural biomass. Ethanol production from non-grain portions of plants is referred to as cellulosic or lignocellulosic ethanol. Lignocellulose is composed of 30% hemicellulose, 44% cellulose and 26% lignin [20]. The structural crosslinking of these polymers presents a physical barrier to hydrolytic enzymes used in the ethanol conversion process, limiting its efficient usage for bioethanol production. Altering cell wall composition, mainly lignin, has long been contemplated as an option to enhance the efficiency of biomass conversion to ethanol [1].

Lignin is a vital component of the plant cell walls. It is responsible for the rigidity required for plant architecture, provides physical protection against pathogens and aids water transport in the xylem [21,22•]. However, during the process of converting biomass into bioethanol, lignin limits the availability of polysaccharides to enzymes, therefore limiting the enzymatic degradability and digestibility of biomass. Maize brown midrib mutants (bm) with an altered lignin biosynthetic pathway have a naturally reduced lignin content and higher digestibility. Two transgenic approaches have successfully mimicked one of these mutant phenotypes (bm3) [23,24]. Piquemal *et al.* [24] used a maize caffeic acid *o*-methyltransferase (COMT) antisense gene construct and showed decreased COMT activity and lignin content in the transgenic maize. He *et al.* [23] obtained similar results using a sorghum *O*-methyl transferase antisense construct in maize, where transgenic plants showed increased digestibility. These studies show the feasibility of using plant transformation to modify the lignin biosynthetic pathway and to alter the lignin profile of maize.

As anticipated, altering plant lignin composition or content can lead to undesired agronomic consequences. Early studies showed that the bm3 mutants were impaired in several agronomical traits; for example, grain and stover yields were reduced by 20% and 17%, respectively (reviewed in [25•]). Additionally, *Arabidopsis* and alfalfa genetically engineered for an impaired lignin biosynthetic pathway showed dwarfism and/or flower color change [26,27]. Currently, more basic research is required to understand the lignin biosynthetic pathway and related areas. The future genetic engineering strategy should be a holistic approach to obtain maize with

maximum digestibility in lignocellulose and minimum reduction in agronomic performance.

Biomass conversion enzymes

Although lignocellulosic feedstocks derived from corn stover could be used for conversion to bioethanol, two major limitations to the process are the costs of transport and processing of biomass. One solution is to produce microbial cellulase enzymes in the plant cells to facilitate the conversion of fermentable sugars *in planta* during the biomass to bioethanol conversion process [28••]. Expression of the catalytic domain of the thermostable 1,4-β-endoglucanase (E1) of *Acidothermus cellulolyticus* in maize [29•] proves the concept that maize can be used as a biofactory for cellulose-degrading enzymes. Even though expression of E1 has not achieved desirable levels, targeting the enzymes to specific subcellular compartments or tissues has shown to be effective in allowing the plants to accumulate higher levels of recombinant enzymes [30,31].

In addition to subcellular targeting of these enzymes, it is also important to express these cell wall degrading enzymes during appropriate developmental stages, rather than over the entire lifetime of the plants. Controlled expression would help to avoid undesired effects on agronomic performance such as lodging or susceptibility to diseases. A senescence-induced promoter might be used to drive cellulase expression in senescing maize. Ideally, the gene should be expressed at the end of the growing season or during post-harvest operations. Other approaches include the use of plant endogenous genes to promote cell wall deconstruction; for example, expansins, a group of hydrogen bond-breaking proteins thought to loosen the cell wall during normal plant growth and development, might be such candidates [32].

Genetic engineering to improve biomass yield

Biomass yield is a complex trait. Although several biotech crop lines engineered for yield enhancement are currently being tested [33], the majority of genes involved in the trait remain elusive. Biomass yield increase and stabilization can be achieved through understanding and enhancing mechanisms such as stress tolerance [34,35••,36,37] and carbohydrate metabolism [38].

Stress tolerance

Enhanced stress tolerance in plants has been achieved mainly through the manipulation of effector genes [39] (e.g. ion transporters, biosynthetic enzymes; see Glossary) and regulatory genes (e.g. transcription factors [40] or signal transduction components [41,42]; see Glossary) from maize itself, other plants or bacterial sources.

Transgenic maize expressing δ-endotoxins from *Bacillus thuringiensis* (Bt) is the classic example of genetic engineering for (biotic) stress resistance. This biotech maize is widely used in North America and constitutes 22 million hectares worldwide [3]. Among the strategies for next-generation insect-resistant crops are the expression of broad-spectrum insecticidal proteins from plants, from bacteria other than *B. thuringiensis* and novel proteins and peptide hormones from insects [43].

Although insect damage can account for as much as 10–20% of crop loss [42] environmental (abiotic) stress has been held responsible for 69% of crop loss [44]. Common denominators are found in response to several stresses, such as the accumulation of reactive oxygen species (ROS) with deleterious effects (e.g. DNA damage and/or impairment of mitochondrial and chloroplast functions). Several excellent reviews addressing genetic engineering for abiotic stress tolerance have been recently published [34,35••] and here we will examine promising approaches centered on plant responses to oxidative stress.

Mitogen-activated protein kinases (MAPKs) are widely associated with the response to biotic and abiotic stress [45], and might be directly linked to the regulation of abscisic acid (ABA)-responsive antioxidant enzymes in maize [46]. Expression of a *Capsicum annum* MAPK in rice and expression of upstream signaling components MAPK kinase kinases (MAPKKKs) from tobacco in *Arabidopsis* yielded increased tolerance to a range of biotic and abiotic stresses [47,48]. Our laboratory has demonstrated the benefits of this strategy in maize, where constitutive expression of *Nicotiana* protein kinase 1, a MAPKKK, enhanced freezing and drought tolerance in transgenic maize plants [41,42]. Other kinases as well as phosphatases also hold much potential in regulating signal transduction in response to stress [45].

De Block *et al.* [49] have successfully prevented the formation of ROS and consequently increased various stress tolerances in *Brassica napus* and *Arabidopsis*. Constitutive expression of the gene coding the antioxidant enzyme super oxide dismutase (SOD) in maize, led to increased tolerance to oxidative damage [39]. More recently, *Arabidopsis* plants with enhanced resistance to several abiotic stresses were obtained by overexpressing not a SOD gene itself, but rather a microRNA involved in the fine regulation of two SOD genes, CSD1 and CSD2 [50].

Much of the study and engineering of plant stress resistance has been in model systems [34]. For instance, a particular class of transcription factors — the dehydration-responsive element-binding protein (DREB)/C-repeat-binding factor (CBF) — interact with the DRE/CRT *cis*-element of many stress-related genes and has been widely studied in *Arabidopsis* [35••]. Constitutive overexpression of *OsDREB1A* and *OsDREB1B* in rice resulted in improved tolerance to drought, high-salt and cold stresses [51]. A recently cloned maize homologue, *ZmDREB1A*,

enhanced cold tolerance when expressed in Arabidopsis [52]. Additionally, the overexpression of the *ZmCRT Binding Factor* increased cold tolerance in maize (reviewed in [40]). Results such as these indicate that many of the mechanisms used to enhance stress response pathways in model systems are applicable to maize and offer a key to reducing biomass and grain yield fluctuations, thereby ensuring steady production for biofuel.

Photosynthesis

As a C4 plant, maize has a compartmentalized photosynthetic system that uses the phosphoenolpyruvate carboxylase (PEPC) as a primary carboxylase [53]. It has been reported that transgenic maize overexpressing PEPC has improved CO_2 fixation rate and compensation point, increased fresh and dry weight, enhanced leaf surface and stomatal density, as well as water stress resistance (reviewed in [54]). Additionally, recent work in transgenic tobacco showed that increased levels of fructose-1, 6-bisphosphatase [55] and sedoheptulose-1,7-bisphosphatase [55,56], two Calvin cycle enzymes, significantly increased dry weight. Interestingly, expression of sedoheptulose-1,7-bisphosphatase also increased leaf area [56].

To adjust to the high planting density currently used in agriculture, modifying plant architecture becomes another way to improve photosynthesis [37]. It has been shown recently in rice that either reducing plant hormone brassinosteroid levels or the amount of the brassinosteroid receptors results in an erect leaf phenotype [57•]. These erect leaf rice plants, obtained either through mutagenesis or genetic engineering, have enhanced biomass production and grain yield under conditions of high-dense planting with no extra fertilization. It is possible that the erect leaf plants are able to enhance photosynthesis by the leaves in the lower part of the plant owing to their altered architecture [58] or are able to reduce the 'shade avoidance syndrome' that is considered to cause stem elongation, early flowering and decreased grain yield in dense planting conditions [59,60].

Grain yield

In 2004, 11% of the maize grain produced in the United States was used to produce ethanol from starch. It is predicted that compared with the 12.87 billion liters of starch ethanol produced in 2004, in 2007 production will reach 20.44 billion liters [1] emphasizing the importance of starch production. As the ADP-glucose pyrophosphorylase (AGP) heterotetramer catalyzes the rate-limiting step in starch biosynthesis, it is usually referred to as a key enzyme in regulating sink strength (see Glossary) in cereal seeds. Deregulation of AGP might lead to increases in plant sink strength and subsequent increases in seed and biomass yield [61–63]. Smidansky *et al.* [61] transformed rice and wheat [63], using the maize Shrunken2 gene Sh2r6hs coding for an AGP large subunit. Compared with control plants, both transgenic wheat and rice plants showed increased seed weight (increased by 38% and 23%, respectively) and increased biomass (increased by 31% and 22%, respectively). Recently a similar strategy in maize produced a 13% to 25% seed weight increase in AGP transgenic plants [64].

Conclusions

Genetic engineering technology presents undeniable potential for future agriculture and biofuel production, as described above. However, the acceptance of biotech-derived crops has met with skepticism and regulatory hurdles in many countries. One major public concern is the control of pollen dissemination for wind pollinated crops such as maize. Plastid genome transformation presents the advantage of limiting transmission of the transgene via pollen while preserving fertility of the plant and allowing higher transgene product production. Although transformation of plastid genomes has been achieved for a few plant species [65], it still remains to be demonstrated in maize. Male sterility offers an alternative approach to control transgene flow, an issue that will probably have a major impact on the development and routine use of biotech crops, in general, and of biofuel-destined crops in particular. Male sterility is a trait that is naturally present in certain lines but it can also be engineered. A recent demonstration of engineered male sterility used chloroplast transformation to produce completely male sterile tobacco plants [66].

It is now clear that multiple transgene strategies need to be developed to tackle complex traits, to engineer metabolic pathways and to combine the expression of different genes. Some studies have demonstrated the feasibility of such technologies [9,67], but more effort is needed to make them both applicable to bioethanol production and acceptable to the public. Indeed, the development of genetically engineered crops raises issues of legislation relating to how these technologies should be regulated and managed. Each country has its own legislation concerning plant biotechnology. Often the regulatory system lags behind the advancement of a technology. An integrated agri-biotechnology system for food, feed and fuel production is likely to be a challenge from the regulatory point of view, but will most certainly be the future for maize if it is to be bred for bioethanol production.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. DOE: US: **The technology roadmap for plant/crop-based renewable resources 2020**. US Department of Energy; 2006.

http://www1.eere.energy.gov/biomass/pdfs/technology_roadmap.pdf.

2. FAO: **FAOSTAT**. Food and Agriculture Organisation of the United Nations; 2004. http://faostat.fao.org/site/291/default.aspx.

3. James C: Preview: global status of commercialized biotech/GM crops, 2006. ISAAA briefs 2006 35. Ithaca, NY: ISAAA.

4. Torney F, Frame B, Wang K: **Maize**. In *Transgenic Crops IV*. Edited by Pua EC, Davey MR. Springer; 2007:73-105.

5. Frame BR, McMurray JM, Fonger TM, Main ML, Taylor KW, Torney FJ, Paz MM, Wang K: **Improved Agrobacterium-mediated transformation of three maize inbred lines using MS salts**. *Plant Cell Rep* 2006, **25**:1024-1034.

6. •• Potenza C, Aleman L, Sengupta-Gopalan C: **Invited review: Targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation**. *In Vitro Cell Dev Biol Plant* 2004, **40**:1-22.
A very thorough review of transgene expression control. Figure 2 of this article is especially useful. Even though published in 2004 it is still a good reference for tissue-specific promoters.

7. Srivastava V, Ow DW: **Biolistic mediated site-specific integration in rice**. *Mol Breed* 2002, **8**:345-350.

8. Halpin C: **Gene stacking in transgenic plants – the challenge for 21st century plant biotechnology**. *Plant Biotechnol J* 2005, **3**:141-155.

9. Ow DW: **Gene stacking through site-specific integration**. In *Genetically Modified Crops, Their Development, Uses and Risks*. Edited by Liang GH, Skinner DZ. Haworth Press, Inc.; 2004:71-100.

10. •• Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr, Hallett JP, Leak DJ, Liotta CL *et al.*: **The path forward for biofuels and biomaterials**. *Science* 2006, **311**:484-489.
A well written review that covers the entire bioenergy field from bioenergy crop production to biorefinery technologies. A must-read article for those who are interested in biofuel production.

11. Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM: **Ethanol can contribute to energy and environmental goals**. *Science* 2006, **311**:506-508.

12. Hill J, Nelson E, Tilman D, Polasky S, Tiffany D: **From the cover: environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels**. *Proc Natl Acad Sci USA* 2006, **103**:11206-11210.

13. Hammerschlag R: **Ethanol's energy return on investment: a survey of the literature 1990-present**. *Environ Sci Technol* 2006, **40**:1744-1750.

14. James MG, Denyer K, Myers AM: **Starch synthesis in the cereal endosperm**. *Curr Opin Plant Biol* 2003, **6**:215-222.

15. Jobling S: **Improving starch for food and industrial applications**. *Curr Opin Plant Biol* 2004, **7**:210-218.

16. Morell MK, Myers AM: **Towards the rational design of cereal starches**. *Curr Opin Plant Biol* 2005, **8**:204-210.

17. Karlsson ME, Leeman AM, Bjorck IME, Eliasson A-C: **Some physical and nutritional characteristics of genetically modified potatoes varying in amylose/amylopectin ratios**. *Food Chem* 2007, **100**:136-146.

18. Jobling SA, Westcott RJ, Tayal A, Jeffcoat R, Schwall GP: **Production of a freeze-thaw-stable potato starch by antisense inhibition of three starch synthase genes**. *Nat Biotechnol* 2002, **20**:295-299.

19. Chih-Ming C, Feng-Shi Y, Li-Fen H, Tung-Hi T, Mei-Chu C, Chang-Sheng W, Hu-Shen L, Jei-Fu S, Su-May Y: **Expression of a bi-functional and thermostable amylopullulanase in transgenic rice seeds leads to autohydrolysis and altered composition of starch**. *Mol Breed* 2005, **15**:125-143.

20. DOE: US: **Breaking the biological barriers to cellulosic ethanol: a joint research agenda**. Edited by Houghton J, Weatherwax S, Ferrell J. Rockville, Maryland: US Department of Energy Office of Science and Office of Energy Efficiency and Renewable Energy; 2006.

21. Baucher M, Halpin C, Petit-Conil M, Boerjan W: **Lignin: genetic engineering and impact on pulping**. *Crit Rev Biochem Mol Biol* 2003, **38**:305-350.

22. • Grabber JH: **How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies**. *Crop Sci* 2005, **45**:820-831.
An interesting review on the properties of cell walls and how these properties affect the hydrolysis of biomass. It presents the concept of using dehydrogenation polymer–cell wall (DHP-CW) complexes of maize primary cell walls as a tool to study how lignin composition, structure and cross-linking affect enzymatic hydrolysis.

23. He X, Hall MB, Gallo-Meagher M, Smith RL: **Improvement of forage quality by downregulation of maize *O*-methyltransferase**. *Crop Sci* 2003, **43**:2240-2251.

24. Piquemal J, Chamayou S, Nadaud I, Beckert M, Barriere Y, Mila I, Lapierre C, Rigau J, Puigdomenech P, Jauneau A *et al.*: **Down-regulation of caffeic acid O-methyltransferase in maize revisited using a transgenic approach**. *Plant Physiol* 2002, **130**:1675-1685.

25. • Pedersen JF, Vogel KP, Funnell DL: **Impact of reduced lignin on plant fitness**. *Crop Sci* 2005, **45**:812-819.
A thorough review on reduced-lignin plants, both mutants and genetically engineered. This article covers more than 10 species: from maize to alfalfa.

26. Reddy MSS, Chen F, Shadle G, Jackson L, Aljoe H, Dixon RA: **Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.)**. *Proc Natl Acad Sci USA* 2005, **102**:16573-16578.

27. Franke R, Humphreys JM, Hemm MR, Denault JW, Ruegger MO, Cusumano JC, Chapple C: **The *Arabidopsis* REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism**. *Plant J* 2002, **30**:33-45.

28. •• Sticklen M: **Plant genetic engineering to improve biomass characteristics for biofuels**. *Curr Opin Biotechnol* 2006, **17**:315-319.
A review dedicated to strategies for altering biomass properties. The focus is on the *in planta* production of biomass conversion enzymes, modification of the lignin content, and increasing plant polysaccharide mass. This review is also a great source of references for a more in depth view of biofuel production related to biomass properties.

29. • Biswas GCG, Ransom C, Sticklen M: **Expression of biologically active Acidothermus cellulolyticus endoglucanase in transgenic maize plants**. *Plant Sci* 2006, **171**:617-623.
Nice example of using the maize crop biomass as a 'green bioreactor' for the production of cellulase enzymes. The catalytic domain of an endo-1,4-β-D-glucanase gene from *Acidothermus cellulolyticus* was expressed successfully in transgenic maize plants.

30. Hood EE: **Where, oh where has my protein gone?** *Trends Biotechnol* 2004, **22**:53-55.

31. Hyunjong B, Lee D-S, Hwang I: **Dual targeting of xylanase to chloroplasts and peroxisomes as a means to increase protein accumulation in plant cells**. *J Exp Bot* 2006, **57**:161-169.

32. Cosgrove DJ: **Growth of the plant cell wall**. *Nat Rev Mol Cell Biol* 2005, **6**:850-861.

33. Dunwell JM: **Transgenic approaches to crop improvement**. *J Exp Bot* 2000, **51**:487-496.

34. Vinocur B, Altman A: **Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations**. *Curr Opin Biotechnol* 2005, **16**:123-132.

35. •• Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K: **Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future**. *Curr Opin Biotechnol* 2006, **17**:113-122.
Surveys 10 years of genetic engineering experiments to improve drought tolerance (1995–2005). Provides three at-a-glance tables addressing strategies based on transgenic expression of effector genes, transcription factors, and signal transduction components. Descriptions of experimental conditions and parameters examined make the tables especially useful.

36. Good AG, Shrawat AK, Muench DG: **Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production?.** *Trends Plant Sci* 2004, **9**:597-605.

37. Sakamoto T, Matsuoka M: **Generating high-yielding varieties by genetic manipulation of plant architecture.** *Curr Opin Biotechnol* 2004, **15**:144-147.

38. Van Camp W: **Yield enhancement genes: seeds for growth.** *Curr Opin Biotechnol* 2005, **16**:147-153.

39. Kingston-Smith AH, Foyer CH: **Overexpression of Mn-superoxide dismutase in maize leaves leads to increased monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase activities.** *J Exp Bot* 2000, **51**:1867-1877.

40. Zhang JZ, Creelman RA, Zhu J-K: **From laboratory to field. using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops.** *Plant Physiol* 2004, **135**:615-621.

41. Shou H, Bordallo P, Fan JB, Yeakley JM, Bibikova M, Sheen J, Wang K: **Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize.** *Proc Natl Acad Sci USA* 2004, **101**:3298-3303.

42. Shou H, Bordallo P, Wang K: **Expression of the Nicotiana protein kinase (NPK1) enhanced drought tolerance in transgenic maize.** *J Exp Bot* 2004, **55**:1013-1019.

43. Ferry N, Edwards MG, Gatehouse J, Capell T, Christou P, Gatehouse AM: **Transgenic plants for insect pest control: a forward looking scientific perspective.** *Transgenic Res* 2006, **15**:13-19.

44. Boyer JS: **Plant productivity and environment.** *Science* 1982, **218**:443-448.

45. Boudsocq M, Lauriere C: **Osmotic signaling in plants. Multiple pathways mediated by emerging kinase families.** *Plant Physiol* 2005, **138**:1185-1194.

46. Zhang A, Jiang M, Zhang J, Tan M, Hu X: **Mitogen-activated protein kinase is involved in abscisic acid-induced antioxidant defense and acts downstream of reactive oxygen species production in leaves of maize plants.** *Plant Physiol* 2006, **141**:475-487.

47. Lee DE, Lee IJ, Han O, Baik MG, Han SS, Back K: **Pathogen resistance of transgenic rice plants expressing mitogen-activated protein kinase 1, MK1, from *Capsicum annuum*.** *Mol Cells* 2004, **17**:81-85.

48. Kovtun Y, Chiu WL, Tena G, Sheen J: **Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants.** *Proc Natl Acad Sci USA* 2000, **97**:2940-2945.

49. De Block M, Verduyn C, De Brouwer D, Cornelissen M: **Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance.** *Plant J* 2005, **41**:95-106.

50. Sunkar R, Kapoor A, Zhu J-K: **Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance.** *Plant Cell* 2006, **18**:2051-2065.

51. Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K: **Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice.** *Plant Cell Physiol* 2006, **47**:141-153.

52. Qin F, Sakuma Y, Li J, Liu Q, Li Y-Q, Shinozaki K, Yamaguchi-Shinozaki K: **Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in *Zea mays* L.** *Plant Cell Physiol* 2004, **45**:1042-1052.

53. Edwards GE, Franceschi VR, Ku MSB, Voznesenskaya EV, Pyankov VI, Andreo CS: **Compartmentation of photosynthesis in cells and tissues of C4 plants.** *J Exp Bot* 2001, **52**:577-590.

54. Jeanneau M, Vidal J, Gousset-Dupont A, Lebouteiller B, Hodges M, Gerentes D, Perez P: **Manipulating PEPC levels in plants.** *J Exp Bot* 2002, **53**:1837-1845.

55. Tamoi M, Nagaoka M, Miyagawa Y, Shigeoka S: **Contribution of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase to the photosynthetic rate and carbon flow in the Calvin cycle in transgenic plants.** *Plant Cell Physiol* 2006, **47**:380-390.

56. Lefebvre S, Lawson T, Fryer M, Zakhleniuk OV, Lloyd JC, Raines CA: **Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development.** *Plant Physiol* 2005, **138**:451-460.

57. • Sakamoto T, Morinaka Y, Ohnishi T, Sunohara H, Fujioka S, Ueguchi-Tanaka M, Mizutani M, Sakata K, Takatsuto S, Yoshida S *et al.*: **Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice.** *Nat Biotechnol* 2006, **24**:105-109.
The authors present a new way of increasing grain yield by altering hormone levels without impairing plant growth and without the use of fertilizer.

58. Sinclair TR, Sheehy JE: **Erect leaves and photosynthesis in rice.** *Science* 1999, **283**:1455.

59. Smith H, Whitelam GC: **The shade avoidance syndrome: multiple responses mediated by multiple phytochromes.** *Plant Cell Environ* 1997, **20**:840-844.

60. Schmitt J, Stinchcombe JR, Heschel MS, Huber H: **The adaptive evolution of plasticity: phytochrome-mediated shade avoidance responses.** *Integr Comp Biol* 2003, **43**:459-469.

61. Smidansky ED, Martin JM, Hannah LC, Fischer AM, Giroux MJ: **Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase.** *Planta* 2003, **216**:656-664.

62. Smidansky ED, Clancy M, Meyer FD, Lanning SP, Blake NK, Talbert LE, Giroux MJ: **Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield.** *Proc Natl Acad Sci USA* 2002, **99**:1724-1729.

63. Smidansky ED, Meyer FD, Blakeslee B, Weglarz TE, Greene TW, Giroux MJ: **Expression of a modified ADP-glucose pyrophosphorylase large subunit in wheat seeds stimulates photosynthesis and carbon metabolism.** *Planta* 2007, **225**:965-976.

64. Wang Z, Chen X, Wang J, Liu T, Liu Y, Zhao L, Wang G: **Increasing maize seed weight by enhancing the cytoplasmic ADP-glucose pyrophosphorylase activity in transgenic maize plants.** *Plant Cell Tissue Organ Cult* 2007, **88**:83-92.

65. Ruiz ON, Daniell H: **Engineering cytoplasmic male sterility via the chloroplast genome by expression of β-ketothiolase.** *Plant Physiol* 2005, **138**:1232-1246.

66. Lu XM, Yin WB, Hu ZM: **Chloroplast transformation.** *Methods Mol Biol* 2006, **318**:285-303.

67. Chen L, Marmey P, Taylor NJ, Brizard J-P, Espinoza C, D'Cruz P, Huet H, Zhang S, de Kochko A, Beachy RN *et al.*: **Expression and inheritance of multiple transgenes in rice plants.** *Nat Biotechnol* 1998, **16**:1060-1064.

Chapter 6: Concluding remarks

6.1. Summary of results

The kinase domain of tobacco mitogen-activated protein kinase Nicotiana protein kinase 1 (NPK1) under the control of a modified 35S promoter was introduced into rice cultivar Nipponbare through Agrobacterium-mediated transformation. The transgene, designated caNPK1 due to it lack of an autoregulatory domain and demonstrated constitutive activity (Kovtun et al. 1998) to differentiate its expression in rice from any effects that might be seen with expression of full-length NPK1.

Twenty-five transgenic events were received from the Iowa State University Plant Transformation Facility. One plant from each event was grown in soil, fifteen analyzed for transgene copy number using Southern blot analysis and twelve of those lines characterized for copy number had transgene expression level assessed by Northern blot analysis and Reverse-Transcriptase PCR (RT-PCR). Two of fifteen lines (13% of lines analyzed) contained a single copy of the *caNPK1* T-DNA cassette. All lines analyzed had low to medium copy numbers (60% 1-3 copies, 40% 4-5 copies). No more than five copies were found in any of the lines analyzed. Transgene expression level as determined by Northern blot analysis correlated well with detection of *caNPK1* transcript by RT-PCR. RT-PCR was more sensitive for revealing low levels of transgene expression. Copy number and gene expression level do not appear to correlate inversely. Plants appeared to grow without gross defects and most were able to set seed within the growth chamber environment.

Four-week old transgenic and wild type plants were treated with moderate (150mM) or severe (300mM) salt stress. Visible signs of leaf damage, electrolyte leakage, malondialdehyde content, sodium ion content within leaves and sodium/potassium ratio did not significantly differ between transgenic and wild type rice when these parameters were measured during the fourth day of salt stress treatment.

Expression of *caNPK1* alters gene transcript abundance in the absence of stress as assessed by microarray analysis. Genes potentially related to flowering and development are downregulated. Biotin biosynthesis and methionine recycling pathway genes appear to be upregulated in the transgenic plants. Thirteen transcripts with functions related to stress are also upregulated in caNPK1-expressing plants. However, quantitative real-time PCR analysis of several known rice MAPKs indicates no significant difference in expression between *caNPK1* transgenics and wild type.

The expression of a constitutively active form of the protein kinase NPK1 in rice produced altered expression of genes, in the absence of stress. The implications of these gene changes in terms of metabolism and response to stress still warrant further investigation. Limited observations of physiological characteristics under salt stress can be expanded in future research. Primarily, measurements of leaf water potential or polyamine content may be measured in *caNPK1* transgenic and wild type rice during the initial phases of salt stress or osmotic shock. An analysis of yield components such as tiller number, panicles per tiller and filled grains per panicle may be undertaken with *caNPK1* transgenic and wild type rice exposed to a lifetime of moderate salt treatment. Additionally, measurements of growth performance, chlorophyll fluorescence and

polyamine levels during low temperature and drought stress can offer parallel data to that described by Shou et al. (Shou et al. 2004a; Shou et al. 2004b).

Transgenic rice expressing a foreignMAP3K provides an experimental system that offers opportunities to study endogenous rice MAPKs and MAP2Ks as well as the general effects of ectopic expression of foreign kinases upon host signaling pathways. Work is currently being done to examine the protein-protein interaction of caNPK1 with putative rice MAP2Ks identified within the KOME database.

References

Kovtun, Y., Chiu, W.-L., Zeng, W. and Sheen, J. (1998). Supression of auxin signal transduction by a MAPK cascade in higher plants. Nature 395: 716-720.

Shou, H., Bordallo, P., Fan, J.-B., Yeakley, J. M., Bibikova, M., Sheen, J. and Wang, K. (2004a). Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc. Natl. Acad. Sci. USA 101: 3298-3303.

Shou, H., Bordallo, P. and Wang, K. (2004b). Expression of the *Nicotiana* protein kinase (NPK1) enhanced drought tolerance in transgenic maize. Journal of Experimental Botany 55: 1013-1019.

Appendix A: Sequence of caNPK1 Cassette within pSHX004

Modified 35s Promoter=nt451-1055

caNPK1 coding domain=nt1056-1895

Double Hemagglutinin tag=nt1902-1958

Nos terminator=nt1896-2275

```
451  gaggatcccc ggccgctcgg gccccccctc gagaagcttc tccaagaata
     tcaaagatac agtctcagaa gaccaaaggg ctattgagac ttttcaacaa
     agggtaatat cgggaaacct cctcggattc cattgcccag ctatctgtca
     cttcatcaaa aggacagtag aaaaggaagg tggcacctac aaatgccatc
     attgcgataa aggaaaggct atcgttcaag atgcctctgc cgacagtggt
     cccaaagatg gacccccacc cacaaggagc atcgtggaaa aagaagacgt
     tccaaccacg tcttcaaagc aagtggattg atgtgatatc tccactgacg
     taagggatga cgcacaatcc cactatcctt cgccccaagc ttgggcccaa
     gcttgggtcg cgccccacgg atggtataag aataaaggca ttccgcgtgc
     aggattcacc cgttcgcctc tcacctttc gctgtactct ctcgccacac
     acacccccctc tccagctccg ttggagctcc ggacagcagc aggcgcgggg
     cggtcacgta gtaagcagct ctcggctccc tctccccttg ctccgtggat
     ccatggcgaa ggctcggaaa gatgacactc cgccaatccg gtggaggaaa
     ggtgaaatga ttggatgtgg tgcttttggt agggtttata tggggatgaa
     tgttgattct ggagagttac tcgctataaa ggaggtttcg attgcgatga
     atggtgcttc gagagagcga gcacaagctc atgttagaga gcttgaggaa
     gaagtgaatc tattgaagaa tctctcccat cccaacatag tgagatattt
     gggaactgca agagaggcag gatcattaaa tatattgttg ggatttgttc
     ctggtggctc aatctcgtca cttttgggaa aatttggatc cttccctgaa
     tctgttataa gaatgtacac caagcaattg ttattagggt tggaatactt
     gcataagaat gggattatgc acagagatat taagggagca aacatacttg
     ttgacaataa aggttgcatt aaacttgctg atttcggtgc atccaagaag
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gttgttgaat tggctactat gactggtgcc aagtcaatga agggtactcc
atactggatg gctcccgaag tcattctgca gactggccat agcttctctg
ctgacatatg gagtgtcgga tgcactatta tcgaaatggc tacaggaaaa
cctccttgga gccagcagta tcaggaggtt gctgctctct tccatatagg
gacaaccaaa tcccatcccc ccatcccaga gcatctttct gctgaatcaa
aggacttcct attaaaatgt ttgcagaagg aaccgcacct gaggcattct
gcatcaaatt tgcttcagca tccatttgtt acagcagaac atcagaggcc
ttacccatac gacgttccag actacgctgg ttacccatac gacgttccag
actacgcttg actgcagatc gttcaaacat ttggcaataa agtttcttaa
gattgaatcc tgttgccggt cttgcgatga ttatcatata atttctgttg
aattacgtta agcatgtaat aattaacatg taatgcatga cgttatttat
gagatgggtt tttatgatta gagtcccgca attatacatt taatacgcga
tagaaaacaa aatatagcgc gcaaactagg ataaattatc gcgcgcggtg
tcatctatgt tactagatcc gatgataagc tgtcaaacat gagaattcct
gcagcccggg ggatccacta gttct                          2275
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Sequencing primers used in primer walk:

NPKseq1: 5’-GCTTAACGTAATTCAACAGA-3’

NPKseq2: 5’-TTCTTGGATGCACCGAATC-3’

NPKseq3: 5’-AGCTCCGGACAGCAGCAGGC-3’

NPKseq4: 5’-TTGCAGTTCCCAAATATCTC-3’

NPKseq5: 5’-CAGCAAGTTTAATGCAACCT-3’

NPKseq6: 5’-ATCATTTCACCTTTCCTCCA-3’

NPKseq7: 5’-TGTTACAGCAGAACATCAGA-3’

NPKseq8: 5’-TATGTTGTGTGGAATTGTGA-3’

Appendix B: Listing of fold difference ratios from Affymetrix Rice Genome Array hybridization

Note: Due to length of array transcript list (>50,000 elements) only genes within the cutoff of RMA q-value < 0.20 are included in this appendix.

gene name	RMA qvalue	RMA Fold Change Ratio (TG:NB)	MAS5.0 qvalue	MAS5.0 Fold Change Ratio (TG:NB)
RPTR-Os-X17220-1_at	5.34E-06	84.94	2.09E-04	245.47
Os.55085.1.S1_at	1.509E-03	-3.14	0.054	-2.75
OsAffx.25720.1.S1_at	2.82E-03	4.54	0.045	7.04
OsAffx.20082.1.S1_at	2.97E-03	54.17	6.12E-06	56.51
Os.56966.1.S1_at	2.97E-03	2.99	6.48E-03	10.14
Os.54747.1.S1_at	3.00E-03	3.50	0.079	4.54
Os.7735.1.S1_at	7.13E-03	3.94	0.281	3.03
OsAffx.16638.1.S1_at	9.52E-03	7.68	0.029	37.85
Os.53831.1.S1_at	9.52E-03	3.03	0.029	10.60
Os.20404.1.S1_at	9.52E-03	1.68	0.048	1.62
Os.50017.1.S1_at	9.52E-03	2.50	0.051	2.06
Os.10659.1.S1_at	9.52E-03	2.90	0.070	22.90
Os.26698.1.S1_a_at	9.52E-03	2.09	0.081	1.91
Os.11411.2.S1_a_at	9.52E-03	-1.25	0.17	-1.25
Os.34982.1.A1_at	9.52E-03	2.46	0.246	2.70
OsAffx.20707.5.S1_at	0.011	1.64	0.40	4.61
OsAffx.5502.1.S1_x_at	0.013	2.35	0.054	2.77
Os.54358.1.S1_at	0.014	1.70	0.024	5.74
Os.50814.2.S1_at	0.016	-1.42	1.40E-03	-1.65
Os.7306.1.S1_at	0.016	5.11	0.048	3.79
Os.54747.1.S2_at	0.020	4.65	5.32E-03	20.40
Os.51557.1.S1_at	0.022	2.70	0.039	2.73
Os.10266.1.S1_at	0.022	11.24	6.48E-03	9.06
Os.4766.1.S1_at	0.022	10.61	6.48E-03	8.22
Os.47329.1.A1_at	0.022	1.46	6.48E-03	1.47
Os.20851.1.A1_x_at	0.022	5.33	0.017	7.19
OsAffx.31316.1.S1_at	0.022	2.03	0.21	4.02
OsAffx.30780.1.S1_at	0.022	1.45	0.26	3.38
Os.5947.1.S1_at	0.022	-1.08	0.62	-1.09
OsAffx.10036.1.S1_at	0.022	1.58	0.14	3.27
OsAffx.2045.1.S1_x_at	0.022	1.06	0.66	1.65
OsAffx.23902.2.S1_at	0.022	3.10	6.48E-03	7.32
Os.10477.1.S1_at	0.023	-1.72	0.14	-1.67
Os.26399.1.S1_at	0.023	-1.58	0.26	-1.56
Os.7727.1.S1_at	0.023	-1.41	0.67	-1.11
Os.53868.1.S1_s_at	0.023	-1.99	0.028	-2.18
Os.54689.1.S1_x_at	0.025	1.39	0.32	2.82
Os.31233.1.S1_at	0.025	84.54	0.48	110.77
OsAffx.7922.1.S1_at	0.025	-1.77	0.56	-1.67
Os.25227.1.S1_x_at	0.025	1.47	0.14	1.55
Os.20183.1.S1_at	0.026	1.48	0.19	1.52
Os.18993.2.S1_x_at	0.026	-1.40	0.45	-1.41
Os.20406.1.S1_a_at	0.026	-1.43	0.56	-1.27
Os.7735.2.S1_x_at	0.027	1.64	0.34	2.06
Os.53355.1.S1_s_at	0.028	-1.84	0.14	-1.91
Os.23660.1.S1_at	0.029	2.12	0.33	1.91
Os.34982.1.A1_x_at	0.031	2.26	0.028	2.35
Os.50502.1.S1_at	0.033	-2.13	0.25	-5.76
Os.23219.2.S1_x_at	0.035	1.14	0.71	1.44
Os.50574.1.S1_at	0.035	-1.38	3.39E-03	-1.60
OsAffx.22999.1.S1_at	0.035	30.54	0.024	15.58
Os.32889.1.S1_at	0.035	19.39	0.40	13.53
Os.10709.1.S1_at	0.035	-1.40	0.36	-1.37
Os.21240.2.S1_at	0.035	6.11	8.92E-04	4.68
Os.23745.1.S1_at	0.035	1.36	0.43	1.26
Os.6256.1.S1_a_at	0.035	-1.36	0.34	-1.56
Os.20638.1.S2_at	0.035	1.09	0.59	1.08
OsAffx.30677.5.S1_x_at	0.035	1.27	0.63	2.72
Os.53868.1.S1_at	0.036	-1.96	0.32	-1.62
Os.27415.1.S1_at	0.038	-1.16	0.26	-1.20
Os.20851.1.A1_at	0.040	9.57	0.015	8.11
OsAffx.23631.1.S1_at	0.042	1.88	0.024	3.08
OsAffx.15929.2.S1_s_at	0.042	-1.24	0.21	-1.24
Os.37457.1.S1_at	0.042	2.09	0.37	2.92
Os.15888.1.A1_at	0.042	-1.08	0.46	-1.07
Os.50765.1.S1_at	0.045	1.83	0.058	2.17

RMA q-values
q<0.05
q<0.10
q<0.15
q<0.20
q>0.20

RMA and MAS5.0 upregulation
>+2
1.5-2
~1.5
downregulation
~-1.5
-1.5-(-2)
>-2

gene name	RMA qvalue	RMA Fold Change Ratio (TG:NB)	MAS5.0 qvalue	MAS5.0 Fold Change Ratio (TG:NB)
Os.8246.1.S1_at	0.046	1.32	0.15	1.31
Os.10910.1.A1_at	0.048	-1.42	0.015	-1.49
Os.52004.1.S1_at	0.049	4.41	0.048	3.85
Os.8480.1.S1_at	0.049	2.37	0.45	2.04
OsAffx.3074.1.S1_at	0.049	-1.34	0.62	-1.22
Os.10477.1.S1_a_at	0.049	-1.71	0.10	-1.68
Os.17912.2.S1_x_at	0.049	-2.95	0.030	-2.28
Os.41946.1.S1_s_at	0.049	1.57	0.40	1.47
Os.53606.1.S1_at	0.049	1.29	0.60	3.16
Os.23316.1.A1_at	0.049	1.13	0.61	1.15
OsAffx.26693.1.S1_at	0.050	-1.11	0.70	-1.15
Os.30575.1.A1_at	0.050	2.14	0.037	5.26
Os.52715.1.S1_at	0.050	1.68	0.067	1.66
Os.10477.2.S1_x_at	0.050	-1.77	0.079	-1.67
Os.34992.1.S1_at	0.050	1.88	0.26	1.84
Os.12381.1.S1_x_at	0.050	11.36	0.35	9.45
Os.53846.1.S1_at	0.050	1.40	0.51	1.81
Os.27064.1.S1_at	0.050	1.26	0.56	1.17
Os.57146.1.S1_at	0.052	2.59	6.48E-03	6.14
Os.5792.1.S1_a_at	0.052	1.55	0.044	1.53
Os.51979.1.S1_at	0.052	1.60	0.26	2.72
OsAffx.25936.1.S1_s_at	0.052	1.73	0.32	2.78
Os.28949.2.S1_at	0.052	-1.54	0.12	-1.67
Os.49194.1.S1_at	0.052	1.19	0.36	2.67
Os.5684.1.S1_at	0.053	1.20	0.28	1.21
OsAffx.16916.1.S1_at	0.053	1.30	0.55	1.55
Os.10659.1.S1_s_at	0.055	8.69	0.044	9.70
Os.18034.1.S1_at	0.055	-2.08	0.30	-2.16
Os.34902.1.S1_at	0.055	-1.13	0.67	-1.13
Os.27815.1.S1_at	0.057	1.29	0.16	1.35
Os.25093.1.A1_at	0.057	1.07	0.64	1.06
OsAffx.1821.1.S1_at	0.059	1.68	0.30	9.75
Os.7215.1.S1_at	0.060	1.27	0.061	1.29
Os.52896.1.S1_at	0.062	1.34	0.066	7.86
OsAffx.17065.1.A1_at	0.063	-1.81	0.29	-1.70
OsAffx.28810.1.S1_at	0.066	1.14	0.62	2.66
Os.54531.1.S1_at	0.066	1.55	0.21	1.52
Os.53027.1.S1_at	0.067	1.32	0.061	5.64
Os.53124.1.S1_at	0.069	-2.81	0.33	-2.92
Os.22910.1.S1_at	0.070	1.34	0.33	1.23
Os.33834.1.S1_x_at	0.071	1.18	0.69	1.11
Os.25227.2.S1_at	0.072	1.40	0.41	1.38
Os.8286.1.S1_at	0.072	-1.29	0.32	-1.30
OsAffx.23143.5.S1_at	0.073	-1.29	0.69	-2.37
Os.17974.1.S1_at	0.073	2.81	0.16	2.35
OsAffx.15443.1.S1_at	0.073	1.63	0.23	3.12
Os.27542.1.A1_at	0.075	-1.13	0.039	-1.39
Os.49826.1.S1_at	0.081	-2.22	0.26	-2.38
Os.17179.1.A1_at	0.081	-1.46	0.14	-1.48
Os.53355.1.S1_at	0.081	-2.14	0.32	-1.90
OsAffx.24621.1.S1_at	0.082	1.11	0.59	4.01
OsAffx.8923.1.S1_at	0.083	1.54	0.56	2.25
Os.9209.2.S1_at	0.083	-2.06	0.54	-1.91
Os.54717.1.S1_at	0.084	1.84	0.39	2.14
Os.73.1.S1_at	0.084	-1.04	0.69	-1.07
Os.7757.1.S1_at	0.084	1.11	0.29	1.08
OsAffx.14131.1.S1_at	0.088	-1.65	0.032	-2.07
Os.51559.1.A1_at	0.088	1.54	0.13	1.47
Os.31233.2.S1_at	0.088	1.79	0.32	1.78
Os.53115.1.S2_at	0.093	-1.13	0.76	-1.11
Os.51535.1.S1_at	0.093	1.96	0.32	1.77
Os.7382.1.S1_at	0.10	-1.96	0.23	-2.68
Os.52519.1.S1_at	0.10	1.35	0.56	1.88
Os.50539.1.S1_at	0.10	-1.18	0.62	-1.17
Os.5811.1.S1_at	0.10	1.11	0.68	1.16
Os.9720.1.S1_at	0.10	1.61	0.31	1.57

gene name	RMA qvalue	RMA Fold Change Ratio (TG:NB)	MAS5.0 qvalue	MAS5.0 Fold Change Ratio (TG:NB)
Os.37647.2.S1_at	0.10	-1.44	0.45	-2.22
Os.21875.1.S1_at	0.10	-1.79	0.26	-1.91
Os.21862.1.S1_at	0.10	2.00	0.024	2.21
Os.53893.1.S1_at	0.10	1.34	0.67	1.18
OsAffx.20320.1.S1_at	0.10	1.39	0.42	1.54
Os.57200.1.S1_at	0.10	-1.16	0.58	-1.15
OsAffx.30041.1.S1_at	0.10	-1.52	0.43	-1.77
Os.51027.1.S1_at	0.10	-1.19	0.56	-1.13
Os.49419.1.S1_at	0.11	1.19	0.81	1.03
Os.35093.1.A1_x_at	0.11	1.64	0.028	2.27
Os.25227.1.S1_s_at	0.11	1.78	0.065	1.58
OsAffx.8602.1.S1_s_at	0.11	1.51	0.19	1.81
Os.15363.1.S1_at	0.11	-1.12	0.49	-1.11
Os.3123.1.S1_a_at	0.11	1.09	0.60	1.06
OsAffx.19483.1.S1_at	0.11	1.11	0.82	-1.14
OsAffx.18587.2.S1_at	0.11	-1.33	0.26	-1.33
Os.51887.1.S1_x_at	0.11	1.17	0.36	1.11
Os.46342.1.S1_at	0.11	-1.22	0.62	-1.14
Os.23883.1.S1_a_at	0.11	1.25	0.45	1.24
Os.37647.1.A1_at	0.11	2.61	6.48E-03	4.29
Os.27386.1.S1_at	0.11	-1.51	0.49	-1.55
Os.49839.1.S1_at	0.11	-1.06	0.61	-1.08
Os.25093.1.A1_x_at	0.12	1.24	0.18	1.25
Os.27584.1.S1_at	0.12	-1.13	0.51	-1.10
OsAffx.23051.1.S1_x_at	0.12	-1.24	0.56	-1.22
Os.54423.1.A1_x_at	0.12	1.38	0.13	1.45
Os.50304.1.S2_at	0.12	1.16	0.26	1.24
Os.54496.1.S1_at	0.12	-1.54	0.40	-1.49
Os.50241.1.S1_at	0.12	1.58	0.15	1.51
Os.53969.1.S1_at	0.12	-1.23	0.44	-1.27
OsAffx.19037.1.S1_s_at	0.12	-1.46	0.49	-1.74
Os.51833.2.S1_at	0.12	1.18	0.48	4.20
Os.4961.3.S1_x_at	0.12	-1.07	0.68	-1.04
OsAffx.6533.1.S1_at	0.13	1.24	0.73	1.15
Os.28427.1.S1_x_at	0.13	-1.47	0.59	-1.48
Os.54496.1.S1_x_at	0.13	-1.40	0.23	-1.51
OsAffx.20644.1.S1_at	0.13	1.16	0.82	1.08
OsAffx.5460.1.S1_at	0.13	1.45	0.48	1.38
OsAffx.31705.2.S1_at	0.13	1.82	0.53	1.93
Os.46638.1.S1_at	0.13	1.10	0.63	1.06
Os.13544.1.S1_x_at	0.13	1.08	0.67	1.07
OsAffx.26155.1.S1_x_at	0.13	1.39	0.51	15.95
Os.35998.1.S1_at	0.13	1.44	0.59	1.31
Os.52736.1.S1_at	0.13	1.96	0.26	2.04
Os.12381.1.S1_s_at	0.14	8.56	0.30	6.96
OsAffx.14372.2.S1_at	0.14	1.12	0.64	2.31
Os.32659.1.S1_at	0.14	-1.09	0.65	-1.06
Os.10521.1.S1_at	0.14	1.13	0.77	1.04
Os.27099.1.S1_at	0.14	-1.12	0.31	-1.14
Os.19044.1.S1_at	0.14	1.93	0.38	1.95
Os.32933.2.S1_x_at	0.14	1.45	0.48	1.32
Os.16088.2.S1_at	0.14	1.07	0.62	-1.55
Os.28605.1.S1_at	0.14	-1.11	0.76	-1.30
Os.27022.2.S1_at	0.14	1.39	0.21	1.74
Os.49976.1.S1_at	0.14	1.70	0.56	2.33
OsAffx.3956.1.S1_at	0.14	-1.16	0.70	1.44
Os.19187.1.S1_at	0.15	-1.47	0.60	-1.27
Os.22269.1.S1_at	0.15	1.38	0.21	1.45
Os.55161.1.S1_at	0.15	1.13	0.79	1.15
Os.24542.1.S1_at	0.15	-1.12	0.76	-1.03
OsAffx.21743.1.S1_s_at	0.15	2.23	0.26	5.76
Os.22269.2.S1_x_at	0.15	1.37	0.39	1.28
AFFX-Os-TrpnX-M_at	0.15	-1.13	0.59	-2.25
OsAffx.24457.1.S1_at	0.15	1.12	0.63	2.14
Os.50704.1.S1_at	0.15	-1.10	0.77	1.07
Os.25002.1.S1_at	0.15	-1.12	0.81	1.10

gene name	RMA qvalue	RMA Fold Change Ratio (TG:NB)	MAS5.0 qvalue	MAS5.0 Fold Change Ratio (TG:NB)
Os.5794.1.S1_a_at	0.15	-1.08	0.69	1.32
Os.47985.1.S1_at	0.15	-1.13	0.73	-1.31
Os.50860.1.S1_at	0.16	1.16	0.57	1.09
Os.55039.1.S1_at	0.16	1.13	0.59	1.95
Os.27022.1.A1_at	0.16	1.44	8.89E-03	1.40
Os.2502.1.S1_at	0.16	1.52	0.072	1.20
OsAffx.6545.1.S1_at	0.16	1.31	0.52	1.35
Os.18676.1.S1_x_at	0.16	-1.24	0.56	-1.28
Os.12054.1.S1_at	0.16	-1.03	0.57	-1.06
Os.32634.1.S1_at	0.16	1.36	0.62	1.41
Os.7534.2.S1_a_at	0.16	1.31	6.48E-03	1.38
Os.33493.1.S1_x_at	0.16	2.28	0.025	2.49
OsAffx.11118.1.S1_s_at	0.16	1.15	0.53	2.13
OsAffx.26673.1.S1_at	0.16	1.37	0.58	1.73
OsAffx.9857.1.S1_at	0.16	-1.14	0.59	-3.30
OsAffx.20605.1.S1_at	0.16	1.04	0.68	-2.11
Os.20614.3.S1_x_at	0.16	1.67	0.38	1.58
Os.50411.2.S1_x_at	0.16	-1.22	0.25	-1.23
Os.4931.1.S1_s_at	0.17	-1.18	0.40	-1.29
OsAffx.12504.1.S1_at	0.17	-1.13	0.36	-1.15
Os.50627.1.S1_at	0.17	1.96	0.49	4.28
Os.52394.2.S1_at	0.17	-1.19	0.61	-1.15
Os.13534.1.S1_at	0.17	-1.29	0.21	-1.29
OsAffx.7013.1.S1_at	0.17	1.25	0.59	1.52
Os.14684.1.S1_at	0.17	-1.89	0.29	-1.90
Os.11770.1.S1_at	0.17	1.34	0.46	1.33
Os.27553.2.S1_at	0.17	1.78	0.20	1.73
Os.54864.2.S1_at	0.17	1.58	0.19	2.57
Os.17475.1.S1_at	0.17	1.27	0.42	1.31
Os.18590.1.S1_a_at	0.17	-1.27	0.59	-1.25
Os.28427.2.S1_x_at	0.17	-1.45	0.84	1.00
OsAffx.31501.1.S1_at	0.17	-1.42	0.15	-1.48
Os.662.1.S1_at	0.17	1.22	0.82	1.03
Os.5129.2.S1_a_at	0.17	1.22	0.59	1.17
Os.55643.1.S1_at	0.17	1.46	0.62	1.33
Os.32933.1.S1_at	0.17	1.40	0.16	1.49
Os.54732.1.S1_at	0.17	-1.55	0.29	-1.60
OsAffx.17142.1.S1_x_at	0.17	-1.48	0.32	-1.41
OsAffx.2810.1.S1_at	0.17	-1.17	0.36	-3.18
OsAffx.4957.1.S1_s_at	0.17	1.46	0.37	1.40
Os.5768.1.S1_at	0.17	-1.38	0.37	-1.53
Os.12023.1.S1_at	0.17	-1.14	0.40	-1.09
Os.7610.1.S1_a_at	0.17	-1.18	0.44	-1.21
Os.46810.1.S1_at	0.17	-1.16	0.54	-1.09
Os.25345.1.S1_x_at	0.17	-1.28	0.56	-1.24
Os.17569.1.S1_at	0.17	-1.31	0.57	-1.59
OsAffx.29396.5.S1_at	0.17	-1.13	0.59	-2.18
Os.8463.1.S1_at	0.17	1.15	0.60	1.12
Os.52586.1.S1_at	0.17	1.21	0.61	1.16
Os.8796.2.S1_a_at	0.17	-1.07	0.62	-1.05
Os.12639.1.A1_at	0.17	1.36	0.64	2.09
Os.5704.1.S1_at	0.17	-1.34	0.67	-1.32
Os.49908.1.S1_at	0.17	1.07	0.69	1.18
Os.52979.1.S1_at	0.17	-1.14	0.74	-1.38
Os.30600.1.S2_at	0.17	-1.22	0.77	-1.04
OsAffx.16034.1.S1_at	0.17	1.03	0.80	-1.05
OsAffx.29842.3.S1_x_at	0.17	1.08	0.83	-1.15
Os.33750.1.S1_at	0.17	-1.16	0.58	-1.18
Os.56358.1.S1_at	0.18	-1.41	0.016	-1.37
Os.8823.1.S1_at	0.18	57.56	0.029	18.65
Os.27569.1.S1_a_at	0.18	1.37	0.13	1.50
Os.52532.1.S1_x_at	0.18	1.09	0.40	1.09
OsAffx.28754.2.S1_at	0.18	1.41	0.55	1.70
Os.6591.1.S1_at	0.18	1.33	0.59	2.24
Os.6798.2.S1_at	0.18	1.36	0.60	2.13
OsAffx.28610.5.S1_at	0.18	-1.13	0.62	-1.89

gene name	RMA qvalue	RMA Fold Change Ratio (TG:NB)	MAS5.0 qvalue	MAS5.0 Fold Change Ratio (TG:NB)
Os.47508.1.A1_at	0.18	1.12	0.63	1.74
Os.31375.1.S2_at	0.18	-1.13	0.65	-1.05
OsAffx.23992.1.S1_at	0.18	1.10	0.68	1.51
Os.6849.1.S2_at	0.18	-1.10	0.74	-1.04
Os.40855.2.S1_x_at	0.18	1.20	0.28	1.48
Os.16886.1.S1_at	0.18	1.45	0.42	1.47
Os.27701.1.A1_at	0.18	-1.14	0.62	-1.05
Os.49360.1.S1_x_at	0.18	-1.06	0.65	-1.10
OsAffx.29888.1.S1_at	0.18	1.09	0.67	1.45
Os.50301.1.S1_at	0.18	-1.13	0.68	-1.08
OsAffx.23378.2.S1_at	0.18	1.29	0.69	1.14
OsAffx.30679.2.S1_x_at	0.18	1.12	0.70	1.22
OsAffx.18542.1.S1_x_at	0.18	-1.10	0.81	-1.08
Os.53326.1.S1_at	0.18	1.35	0.41	1.48
Os.43929.1.S1_s_at	0.18	56.36	0.048	13.54
Os.16099.1.S1_at	0.18	2.10	0.16	2.58
Os.51724.1.S1_at	0.18	1.36	0.40	1.27
Os.48919.1.S1_at	0.18	1.40	0.44	1.31
Os.9853.4.S1_x_at	0.18	-1.24	0.64	-1.23
Os.53818.1.S1_at	0.18	-1.12	0.71	1.71
OsAffx.19683.1.S1_at	0.18	-1.11	0.79	-1.28
Os.17900.1.S1_s_at	0.18	1.54	0.17	1.64
Os.17002.1.S1_at	0.18	-1.11	0.26	-1.11
Os.7822.1.S1_at	0.18	-1.24	0.29	-1.18
Os.2436.1.S1_at	0.18	1.61	0.59	1.64
OsAffx.5656.1.A1_at	0.18	-1.14	0.60	-1.92
Os.29987.1.S1_at	0.18	-1.04	0.75	-1.24
OsAffx.4595.1.S1_at	0.18	-1.15	0.81	-1.15
Os.9303.1.S1_at	0.18	-1.34	0.40	-1.37
Os.53855.1.S1_at	0.18	1.33	0.56	1.38
Os.28427.1.S2_a_at	0.18	-1.54	0.62	-1.75
Os.42436.1.S1_at	0.18	-1.26	0.63	-1.41
OsAffx.17122.2.S1_at	0.18	1.13	0.56	3.53
Os.623.2.S1_x_at	0.19	-1.08	0.56	-1.10
Os.47600.2.S1_x_at	0.19	-1.09	0.54	-1.14
Os.8568.1.S1_at	0.19	-1.71	0.29	-1.90
OsAffx.4205.1.S1_x_at	0.19	2.31	0.21	2.51
Os.32945.1.S1_at	0.19	1.12	0.30	1.15
Os.19971.1.S1_at	0.19	1.95	0.36	1.89
Os.53626.1.S1_at	0.19	-1.14	0.43	-1.46
Os.48948.1.S1_x_at	0.19	1.83	0.52	5.05
Os.37577.1.S1_s_at	0.19	-1.44	0.55	-1.36
Os.17691.1.S1_at	0.19	-1.09	0.56	-1.15
Os.23660.1.S2_at	0.19	1.38	0.56	1.56
Os.8275.1.S1_at	0.19	1.10	0.62	1.16
Os.56097.1.S1_at	0.19	-1.14	0.64	-1.69
Os.25740.1.S1_at	0.19	1.16	0.67	1.51
Os.50223.1.S1_at	0.19	-1.08	0.76	-1.23
Os.2362.2.S1_x_at	0.19	-1.23	0.62	-1.28
Os.53672.1.A1_at	0.19	1.77	0.32	1.73
Os.24397.1.S1_at	0.19	1.22	0.47	1.53
Os.4441.1.S1_at	0.19	-1.05	0.53	-1.11
OsAffx.3189.1.S1_at	0.19	-1.06	0.80	1.20
Os.20079.1.S1_s_at	0.19	-1.36	0.38	-1.29
Os.7391.1.S1_x_at	0.19	-1.07	0.64	-1.07
Os.11442.1.S1_at	0.19	1.22	0.43	1.23
Os.40360.1.S1_at	0.19	1.28	0.62	2.45
Os.52216.1.S1_at	0.19	1.37	0.42	1.39
Os.20066.1.S1_at	0.19	-1.29	0.38	-1.16
Os.49104.1.A1_at	0.20	1.16	0.60	1.14
Os.17640.1.S1_at	0.20	1.18	0.30	1.20
Os.23685.1.S1_x_at	0.20	-1.07	0.69	-1.75
Os.5111.1.S1_at	0.20	-1.27	0.56	-3.32
Os.43888.1.S1_at	0.20	-1.13	0.59	-2.00
Os.7854.1.S2_at	0.20	-1.22	0.65	-1.15
Os.14932.1.S1_at	0.20	1.23	0.53	1.20